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THE UNIVERSITY OF ALBERTA

MANIPULATION AND CRYOPRESERVATION OF EMBRYOS OF THE DIABETIC (BB) RAT

By

(C) Lorraine Cecilia Bruch (Skleryk)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

In

Experimental Medicine

Department of Medicine

EDMONTON, ALBERTA

SPRING 1987

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **MANIPULATION AND CRYOPRESERVATION OF EMBRYOS OF THE DIABETIC (BB) RAT** submitted by **LORRAINE CECILIA BRUCH (SKLERYK)** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE**, (Experimental Medicine).

.....

Supervisor

.....

.....

Date: Apr 15/87, 1987.

This text is dedicated to

my entire family.

ABSTRACT

The BB rat presents an excellent animal model for the study of the etiology, pathophysiology and complications of human insulin-dependent diabetes mellitus. Low temperature banking of this valuable rat embryo would ensure the strain would not be lost to disease, natural hazards or genetic drift and would reduce the time and cost of maintenance of the colony.

Eight-cell and blastocyst BB rat embryos were frozen slowly in the presence of 2 M Me_2SO to either -40°C or -75°C, stored in liquid nitrogen and then warmed rapidly or slowly, respectively. Viability on thawing was asseessed using the fluorescein diacetate assay, in vitro culture and in vivo embryo transfer. Thirty-one per cent of 8-cell and 74% of blastocysts developed in culture after slow cooling to -40°C, storage in liquid nitrogen and rapid thawing. Two and a half per cent of 8-cell and 13.5% of blastocysts developed into normal fetuses after transfer to pseudopregnant recipients following this same freeze-thaw protocol. There was no significant difference between the freeze-thaw protocols as measured by in vitro viability assays. Blastocysts are the better stage of development for in vitro manipulation, cryopreservation, culture and in vivo embryo transfer in the BB rat.

The ability of the 8-cell BB rat embryo to develop in culture after exposure to 2 M Me_2SO was significantly reduced. Rat embryos cultured in tubes (batch culture), developed better than those cultured under

paraffin oil. The optimal pH for *in vitro* development of preimplantation BB rat embryos was 7.27-7.32.

The ability of the BB rat blastocyst to fluoresce in the presence of the dye, fluorescein diacetate, was a good indication of viability.

Since the establishment of an embryo cryobank for the BB rat depends on the success of *in vivo* transfer, further research must be focused in this area.

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GLOSSARY OF TERMS

PMSG	Pregnant Mare Serum Gonadotrophin
HCG	Human Chorionic Gonadotrophin
FDA	Fluorescein Diacetate
BMOC-3	Brinster's Medium for Ovum Culture
FCS	Fetal Calf Serum
PBS	Dulbecco's Phosphate Buffered Medium
PB1	Modified Dulbecco's Phosphate Buffered Medium
Me2SO	Dimethyl Sulfoxide

I

INTRODUCTION

The spontaneously diabetic Wistar rat, (BB rat), was discovered in 1974 at the Biobreeding Laboratory in Ottawa (1). The characteristics of the diabetic syndrome in the BB rat closely resemble those found in insulin-dependent diabetes mellitus in humans (1-4). The disease occurs spontaneously in nonobese rats derived from an outbred Wistar line. Both sexes are affected and the onset of overt diabetes occurs between 60 and 120 days (3). Clinical manifestations include weight loss, polyuria, polydipsia, elevated non esterified fatty acid levels, markedly depressed insulin levels and elevated glucagon levels (2). Genetic and immune factors are involved in the etiology of the disease. A significant symptom is the occurrence of intense insulitis with selective beta cell destruction (2,3). The diabetic syndrome is inherited as an autosomal recessive trait in the BB rat (2). Recently, various diabetic complications have been reported in this animal model including neuropathy (3) and slow early diabetic retinopathic changes (5). This animal provides an excellent model for the study of etiology, pathophysiology and complications of diabetes potentially applicable to man (3).

Since their discovery, many studies have been carried out using the BB rat and these studies have addressed problems pertaining to insulin-dependent diabetes mellitus in man (6-9).

Because of the early onset of diabetes in the BB rat, daily insulin therapy is required for all animals of breeding age. The strain is also very susceptible to infections by common varieties of bacteria,

mycoplasma and viruses. These rat are maintained in a "germ free" environment in Ottawa (3). Maintenance of the colony is expensive and time-consuming. Diabetic females are prone to stress and often have a reduced fertility. Litters are small and pups often must be fostered, as the diabetic mother cannot nourish the litter sufficiently (10). It is also expected that the characteristics of the diabetic syndrome of the BB rat will drift with time in different breeding colonies (3).

For these reasons, this animal model of insulin-dependent diabetes is an excellent candidate for low temperature embryo banking. Valuable strains of mice have been stored for several years in 15 institutions throughout the world (11). Stocks of mutant mice have been reestablished after storage in liquid nitrogen for up to 11 years (12). The benefits of low temperature storage are many. In the frozen state, embryos are not affected by disease, genetic drift or environmental accidents (11). With frozen embryos as a back up, strains could be maintained at a reduced cost and in less space (11).

Rat embryos have been successfully cryopreserved using techniques developed for the mouse embryo (13,14). In both studies, investigators expressed the need for development of better in vitro culture techniques for the rat embryo. There is also a need for improvement of the present techniques for induction of pseudopregnancy and transfer of embryos to foster mothers.

To establish a low temperature bank for the BB rat embryo several techniques must be optimized. Sufficient numbers of embryos are required. Suitable in vitro culture techniques and media must be available for initial assessment of viability before and after cryopreservation. Optimal freezing and thawing protocols are necessary,

specific for the BB rat embryo. And finally, reliable methods of embryo transfer must be developed in the rat model.

Because these techniques have been well developed for the mouse, this animal has been used extensively in mammalian embryo research (6,15-18). Large numbers of eggs are easily obtained by hormonal induction of ovulation in the mouse (19). Reports on superovulation for the rat, however are not as promising, as large numbers of abnormal eggs are often recovered on isolation (20-22). Hormonal induction of ovulation in the rat produces inconsistent numbers of normal eggs and most investigators prefer to use natural mating (13,14).

Tissue culture techniques and nutritional requirements for preimplantation mouse embryos in vitro have been extensively investigated (15,16,23-25). Two-cell mouse embryos are routinely cultured through to the expanded blastocyst stage (15,26) and some inbred strains develop in culture from fertilized ova through to the blastocyst stage (16). For the rat, there is no development past the 4-cell stage in vitro (27), although 8-cell embryos develop into expanded blastocysts in culture (13,27,28). There is limited knowledge regarding the in vitro nutritional requirements for all stages of the preimplantation rat embryo.

In order to establish a low temperature embryo bank for the BB rat, the development of optimal techniques for in vivo transfer of the cryopreserved embryos on thawing is essential. In vivo transfer techniques have been quite successful in the mouse. Several investigators report a survival rate of 30% to 54% after transfer of frozen thawed embryos (12,29,30). Again, these techniques for the rat embryo have proven to be more difficult (13,14). Induction of the state

of pseudopregnancy, which is essential in rodents for successful implantation of the transferred embryos (31,32), could be one of the reasons for low success following the transfer procedure (14). The actual technical aspects of embryo transfer in the rat is more complex than for the mouse. Transfer of embryos younger than 8-cell is more difficult in the rat (33,34). It is possible that the rat uterus is more sensitive to the trauma imposed during transfer of embryos. The precise synchrony of embryo stage and developmental stage of the uterus is also critical (32,34).

All of these problems must be investigated before successful *in vivo* transfer of the BB rat embryo can be consistently carried out.

The purpose of this study was:

- to consider hormonal induction of ovulation in the BB rat as a method for obtaining consistent numbers of embryos.
- to investigate two different freezing and thawing protocols for the BB rat embryo.
- to assess the application of the fluorescein diacetate dye assay as an alternate *in vitro* test of viability after cryopreservation.
- to examine *in vitro* manipulation and culture techniques for BB rat embryos.
- to examine the technique of *in vivo* transfer of the BB rat embryo as the ultimate viability assay.

The final goal is to establish a low temperature embryo bank for the BB rat embryo. This would ensure that the strain would not be lost to genetic drift, disease or natural hazard. Transport of this strain to and from other laboratories would be much more convenient and the strain would be more available for research which may eventually lead to a better understanding of the etiology, pathophysiology and complications of human insulin-dependent diabetes.

II

LITERATURE REVIEW

An Overview of Mammalian Embryo Research

Although first reports of manipulation and transfer of fertilized ova were reported in the 1890's, the main interest and expansion of knowledge has occurred in the last thirty years. With the present day interest in *in vitro* fertilization and embryo transfer in humans to overcome infertility barriers, the wealth of knowledge in the past five years has really expanded.

Basic manipulation and handling techniques were first developed in the amphibian embryo (35). Mammalian embryos posed new problems, namely availability, means of securing embryos in the desired stage of development, methods of maintaining viability outside of the maternal environment and development of morphological criteria which could be used to classify the embryos as normal or abnormal.

The first reported successful transfer of fertilized rabbit ova from one strain to another was in 1891 by Walter Heape (36). He is considered by many to be the pioneer of mammalian embryo experimentation. The main question he was trying to answer was whether the uterine environment influenced an embryo's phenotype. Heape defined the terms in use today to describe the phases of the estrus cycle (36).

It was shortly after this that the first attempts to study mammalian embryonic development outside the maternal reproductive tract were reported by Schenk and Onanoff in 1893. They reported limited cleavage

in the early rabbit and guinea pig embryo *in vitro*, however, their techniques could never be repeated (37).

In 1929, Lewis and Gregory observed cleavage of fertilized rabbit embryos from the pronuclear stage to the blastocyst stage *in vitro* (38).

The first manipulations and successful transfer of rat embryos were reported by Nicholas in 1933 (39). In his experiments, he transferred 2- and 4-cell embryos from albino donors to already pregnant female hooded recipients. His main interest was egg viability and their capacity for continued development after manipulation. Nicholas obtained very good results, 80% survival, if the donor embryos were at an earlier stage in development than the recipient's own embryos at transfer.

In the same year, Defrise carried out a series of experiments using all preimplantation stages of the rat embryo and various media combinations to observe the appearance, size and developmental behavior of the different stages *in vitro* (40). Although he only observed a single cleavage division *in vitro* with one specific recipe, he did make several contributory observations. Defrise commented on abnormal criterium of development and described the process of degeneration when an embryo was exposed to an unsuitable *in vitro* environment. He noted that: "...eggs of rats are extremely sensitive to variations in temperature, osmotic pressure and the hydrogen ion concentration of the medium..." (40).

Several investigators made significant contributions to the development of *in vitro* culture techniques. Most of these techniques were defined for the preimplantation stages of the mouse embryo. In 1949, Hammond cultured 2-, 4- and 8-cell mouse embryos in a saline solution with glucose and thin egg white with considerable success. He

observed no development of 2-cell embryos, limited development of 4-cell embryos and good development of 8-cell embryos to the blastocyst stage (41). Soon after, Whitten (26,42) substituted bovine serum albumin (BSA) for egg white and observed excellent development of 8-cell mouse embryos to blastocysts in culture. A very important discovery was that the addition of lactate to the culture medium enhanced the development of late 2-cell mouse embryos to the blastocyst stage (42).

In 1963, Brinster's famous paper came out describing precisely the requirement for *in vitro* development of the preimplantation mouse embryo (15). He also introduced the method of culture most used today, that of microdroplets under paraffin oil. With this method he obtained 70-100% development of 2-cell mouse embryos to blastocysts. By 1971 Brinster made a few important changes to his original medium and the result is what many investigators use today, Brinster's Medium for Ovum Culture (BMOC-3) (24).

Less work has been done on *in vitro* culture techniques for the rat embryo. Folstad, Bennet and Dorfman employed the techniques of Brinster in 1969 and tested various media with the culture of 2,4 and 8-cell rat embryos (28). They obtained good results with 90% of the 8-cell embryos developing to morula or blastocysts after culture for 24 hours. The preimplantation stages of the rat embryo were studied again by Mayer and Fritz in 1974 (27), but they used a medium developed by Biggers *et al*. in 1971 (37). They reported no cleavage of the 4-cell rat embryo and 80% development of 8-cell to the blastocyst stage after 24 hours in culture (27). They substituted BSA with fetal calf serum (FCS).

The problems of availability of mammalian embryos was partially overcome by the discovery in 1927 that mice and rats could be induced to

superovulate by hormones secreted by the anterior pituitary (43). In 1944, Rowlands reported the production of ovulation in immature rats with administration of mare serum gonadotrophin and chorionic gonadotrophin given 48-72 hours apart (44). The action of these hormones was the same as that for the hormones secreted by the anterior pituitary in stimulating first, follicle growth and then release of matured ovum. This breakthrough enabled investigators to obtain large numbers of eggs for study in a controlled situation.

In 1953, McLaren and Biggers (45) obtained live mice after transfer of blastocysts obtained at the 8-cell stage from superovulated females and then cultured using Whitten's procedure for 48 hours. They reported no difference between control and cultured embryos after transfer.

As the techniques were being developed for the manipulation, culture and transfer of mammalian embryos, a new field of research was emerging, that of low temperature preservation or cryopreservation. The significance of this science to mammalian embryology was quickly realized. Storage of embryos at a subzero temperature would be valuable in maintaining mutant strains of laboratory animals.

In 1953, Audrey Smith was the first to report successful low temperature storage of single cell fertilized rabbit eggs (46). One per cent of eggs frozen to -79°C in 15% glycerol continued division in culture. Sixty-nine per cent survived three days at -15°C provided they remained supercooled.

There was a lull in this area of cryobiology for several years. During this time knowledge was being accumulated about the response of different cell types exposed to subzero temperatures. Information about factors such as cell size, permeability, rates of cooling and warming,

cryoprotective agents and final storage temperature were all important in characterizing the conditions for obtaining optimal survival for each cell type (31,48).

It was in 1972 when two separate papers appeared reporting the successful cryopreservation of mouse embryos. Whittingham, Leibo and Mazur, studied cooling and warming rates and the suspending medium effects on 8-cell mouse embryos (29). They found that the optimal cooling rate was much slower than for any other cell type frozen to date. The optimal warming rate was also very slow. Wilmut made the same observations in another laboratory (17).

Since these first reports, several theories on mechanisms of injury to the embryos during freezing and thawing have been proposed (49-51). The mechanisms of the protective action of several cryoprotective solutions have also been defined. The successful low temperature storage of several other mammalian embryos have since been reported including the rabbit and rat although survival rates are not as high as those for the mouse embryo (47).

Rat embryos were successfully cryopreserved in 1975 by Whittingham (13). Optimal survival was achieved with cooling and warming rates similar to those reported for mouse embryos. Both *in vitro* (61%) and *in vivo* (9%) survival rates were low compared to those obtained in mice.

In 1980, Wood and Whittingham froze 8-cell and blastocyst rat embryos (14). Their *in vivo* survival rates for frozen-thawed embryos were no different than control groups, however, both were low. These investigators expressed the need for a better understanding of *in vitro* requirements and embryo transfer in the rat.

Because each individual mammalian embryo has specific nutritional requirements to survive in vitro (24), there remains a need for better understanding of the differences between species and changes which occur during their early development (48). Some of the problems still to be overcome besides the in vitro requirements of the rat embryo are, the ability to obtain a maximum number of embryos, determination of the most appropriate stage for freezing and appropriate and reliable viability assays.

Obtaining Sufficient Numbers of Embryos

There are two methods for obtaining mammalian embryos, the first is hormonal induction of ovulation and the second is by natural mating.

A. Hormonal Induction of Ovulation

One of the problems in mammalian embryology studies is availability of embryos in the appropriate stages of development. It would be advantageous to obtain large numbers of embryos from a single animal. In 1927, Smith and Engle discovered that the anterior lobe of the pituitary secreted a hormone which caused precocious maturity of the ovary in immature mice and rats (43). They also observed that the transplantation of the pituitary from a mature animal to an immature one often caused superovulation. As many as 48 normal ova were found in rats only 26 days old.

In 1930, the action of pregnant mare serum gonadotrophin (PMSG) was described by Cole and Hart. This hormone mimics the anterior pituitary

follicle stimulating hormone FSH and stimulates the growth of ovarian follicles (52). The action of human chorionic gonadotrophin (HCG) described by Aschheim and Zondek in 1928, stimulated rupture of mature follicles and therefore mimicked the luteinizing hormone LH secreted by the anterior pituitary (52). With this knowledge, the use of these hormones for inducing ovulation and superovulation in immature rats and mice began.

In 1944, Rowlands reported the production of ovulation in immature rats with administration of 10 IU PMSG followed 48-72 hours later by 10 IU of HCG (44). With these doses he was able to obtain 10 embryos per rat consistently. With a dose of 30 IU PMSG and 20 IU HCG he obtained an average of 26 embryos per rat. His data showed that the amount of PMSG and time between injections of the two hormones were important in producing ovulation. A small amount of HCG given after an adequate dose of PMSG produced ovulation in 100% of the animals. The optimal time between injections was 48-56 hours for 90-100% ovulation. He observed no ovulation with a single dose of PMSG up to 60 IU.

The use of hormones to stimulate ovulation in mature animals was reported by Fowler and Edwards in 1957 (53). They used relatively low doses of the hormones administered 40 hours apart. Fewer hormonally ovulated female mice became pregnant (75%) after mating than those mated naturally (90%). Even with low doses of these hormones they obtained approximately 24 eggs per female which is significantly more than that ovulated in a normal estrus cycle. Immature mice ovulate up to 40 eggs per female when superovulated and Fowler and Edwards showed that mature animals yield fewer ova when ovulation is hormonally induced (53).

Another report came out a year later by Zarrow, Caldwell, Hafez and Pincus (54). In this study they were able to produce superovulation in immature rats using 30 IU of PMSG followed 56 hours later by 5 to 20 IU of HCG. The maximum number of ova recovered per rat was thirty.

In the early 1960's, Zarrow and Wilson did some comparisons of the ovulatory response to hormones in immature mice and rats (55). They found that the optimal age for maximum number of ova released in the rat was 23 days of age. Thereafter, higher doses of the hormones were required to get a superovulatory response. The response produced 100% ovulation in rats between the ages of 21 and 40 days old, but decreased to 50-65% after this. The decrease in numbers of ova ovulated after a certain age was attributed to a change in sensitivity of the follicles to the gonadotrophins (55). In 50 day old rats, these workers obtained 15-21 ova per rat and by 60 days of age 70% of females ovulated with an average of 18.3 oocytes per rat. They felt the number of ova ovulated was not as dependent on the dose of HCG given as on the PMSG dose administered. In every experiment the mouse ovulated more ova per female than the rat.

These two investigators also reported no ovulation in rats given a single dose of PMSG, and 60 IU of this hormone produced cystic follicles indicating overstimulation (56).

Despite the obvious benefits of superovulation, there have been reports indicating that hormonal induction of ovulation in immature rats causes a reduced fertility in these rodents. One report in 1950, suggested that this decreased fertility may be due to an incomplete state of estrus, a lowered rate of fertilization and abnormal development in some of the oocytes (57). Lately, there have been many reports of

abnormal ovulation and early embryonic mortality when superovulatory doses of PMSG are administered to immature rats (20-22). Investigators conclude that the loss of embryos during the first 36 hours after ovulation is due to a failure of the ovaries to provide a suitable hormonal environment rather than defects in oocyte maturation, ovulation or fertilization.

B. Natural Mating

In most papers cited and in several breeding colonies, mature female mice and rats are placed with known breeders and are checked daily for the presence of a copulation or sperm plug (58).

The vaginal milieu shows a very clear picture of the stages of the estrus cycle in the mouse and rat (59,60). In 1941, Young et al. studied the complete estrus cycle as it relates to the vaginal epithelium in the albino rat (60). They observed that the beginning of heat usually coincided with the beginning of complete cornification of the epithelial cells in the smear. Their study gives the percentage of epithelial cells, leukocytes and cornified cells and the correspondence of these to breeding as well as appearance of the ovaries.

A complete description of the stages of the estrus cycle of the rat is found in Bennet and Vickery (59). Estrus is indicated by a marked squamification and cornification of the cells and total disappearance of leukocytes. The end of estrus is marked when the cornified cells are sloughed off and the smear shows many leukocytes. During the 24 hours prior to the next estrus, the epithelial cells become abundant and their size and degree of pyknosis increases and the number of leukocytes

decreases markedly. Since estrus occurs between 4 and 10 pm in the rat and lasts approximately 14 hours with ovulation occurring at 3 am, the female is most receptive in the first 3 hours of estrus (59).

Vaginal smears are an excellent indication of estrus and mature females mated with proven males are fertilized with a high incidence (59). It is best if the males are allowed 3 days between matings.

In either method, the day designated as day 1 is the morning following mating. One to eight-cell embryos can be obtained from the oviducts on days 1 through 4, and blastocysts from the uterus on day 5. A more detailed discussion of normal and abnormal embryos will be undertaken in the following section.

Advances in Isolation Techniques and Embryo Manipulation

An enormous amount of knowledge on the development and morphology of the preimplantation stages of the mammalian embryo has accumulated since Defrise's observations of rat embryos in 1933 (40). He reported size, shape and morphological appearance of rat embryos.

Work on mammalian ova and embryos has increased tremendously especially with development of techniques for *in vitro* studies which have contributed information improving our understanding of morphology and functional aspects of early embryos.

The mammalian oocyte is ovulated with a zona pellucida and cumulus oophorous. After fertilization, cleavage from 2- to 4-cell and 4- to 8-cell is relatively but not entirely synchronous between the blastomeres. Beyond the 8-cell stage, division of individual blastomeres

becomes more and more asynchronous. At the morula stage cells become compacted and tight junctions are formed between them (25,47). The blastocoele is formed and the blastocyst expands by active transport of fluid into the cavity. Cavitation is timed by cleavage division rather than by cell numbers (25). Evidence is given for this because if some, or all but one, of the blastomeres are destroyed in the 4- or 8-cell stage of the mouse embryo, the rest form a cavity (25). Expanded blastocysts consist of a single peripheral layer of cells which will form the trophoblast and an inner cell mass which will form the embryo (47). Formation of the blastocyst is the first overt sign of cellular differentiation, as two different cell types exist (25). Later stages of expansion show marked thinning of the zona. Hatching from the zona varies among species as does expansion and compaction (47).

One definition of a normal embryo is: those embryos having the right number of morphologically normal blastomeres (61). On time cleavage of the preimplantation mammalian embryo is also an indication of normality. In the rat, the first cleavage division occurs between 0 and 24 hours after ovulation, the morula stage is reached between 72 and 84 hours after ovulation and the blastocyst is formed between the 4th and 5th day post-ovulation (27).

The ability to separate abnormal from normal cleavage when observing the progress of development *in vitro* is extremely important for obvious reasons. Defrise (40) noted abnormalities in cleavage divisions among the blastomeres of rat embryos which had developed with blastomeres of varying sizes; these embryos containing irregularly sized blastomeres are now referred to as having undergone fragmentation (58). Another contribution Defrise made were his observations on the response of the

embryos to various osmotic, temperature and pH changes of the handling medium. When embryos were exposed to unsuitable media he defined a criterion for morphological degeneration. He noted a shrinking of the cytoplasm with intracellular precipitation and formation of dark spots, clouding of the cellular content, swelling, cessation of mitosis and fusion of the blastomeres. Defrise also reported that the zona remained intact almost until implantation and was lost by swelling of the blastocyst and dissolution of the membrane (40).

Today, the abnormal development of preimplantation stages of mammalian embryos has been verified and defined (58). The morphological criterion for abnormal cleavage include: fragmentation, which is the occurrence of blastomeres of grossly unequal size, or the appearance of more blastomeres than appropriate for the time course of development; unusual configuration of blastomere arrangement; and vesiculation cavities which are not blastocoele cavities. These morphological criteria have been established along with the development of appropriate <u>in vitro</u> culture techniques.

The embryo is a delicate organism and is subject to injury and death by the slightest change in temperature, pH or osmotic pressure (40,62). Isolation techniques have improved tremendously and all stages of preimplantation embryos are readily obtained. In the rat, the 1- to 8-cell stages can be flushed from the oviducts, and the 8-cell embryo to blastocyst stage from the uterus. Sterility is essential in all techniques as described by several investigators (58,62,63). Siliconization of glassware is also important as embryos stick if albumin is not included in all medium (63). The handling medium is also of utmost importance. Dulbecco's phosphate buffered saline (PBS) was

developed in 1954 by Dulbecco and Vogt (64). In 1969, Whittingham and Whitten altered this medium with the addition of lactate, pyruvate and bovine serum albumin (65). This medium, (PB1), is widely used for short term *in vitro* studies and as the suspending medium for cryopreservation (13,17,18).

Review of Cryopreservation

Audrey Smith in 1953, showed that fertilized rabbit ova could survive exposure to subzero temperatures if in the presence of 15% glycerol (46). The possibility of freezing mammalian embryos had been initiated. The task was now set before cryobiologists and embryologists to gain knowledge in this new field.

The area of cryobiology is very complex. Individual cell types respond differently to cooling and warming rates. They respond differently to cryoprotective additives, suspending medium, and intermediate cooling temperatures prior to immersion in liquid nitrogen. The development of these techniques or protocols for the mammalian embryo is complicated by the stage of development chosen for the freezing procedure. Cell volume, size and permeability characteristics all govern a specific cell's response to addition and dilution of cryoprotective solutes as well as to freezing. There are also species differences as embryos of different species respond independently to *in vitro* manipulative procedures. Considering all of these factors the breakthrough by Whittingham, Liebo and Mazur and by Wilmut in 1972 (17,29) was significant to the area of embryology as well as to cryobiology. Methods for freezing and thawing embryos have not changed

drastically since these first reports but much knowledge has been gained in understanding the physiochemical and physical processes which occur during these events. These techniques have also led to a better understanding of the development of preimplantation stages of mammalian embryos.

In 1975, Whittingham was the first to report cryopreservation of 2-, 4- and 8-cell rat embryos to -196°C for up to 3 months (13). He used dimethyl sulfoxide (Me_2SO) as the cryoprotectant in PBl medium and studied various cooling and warming rates. The optimal cooling rate was similar to that required for survival of mouse embryos (17,29), between 0.6 and 1°C/min. The optimal warming rate was also very slow. He assessed their viability using both in vitro culture and in vivo transfer to pseudopregnant recipients. Sixty-one per cent of the 8-cell rat embryos developed into blastocysts and 9% of the 8-cell embryos transferred to foster mothers developed into normal young.

Whittingham suggested that the results would improve if better methods for embryo culture and transfer and for the induction of pseudopregnancy were developed (13).

The most recent work with cryopreservation of rat embryos was a study by Wood and Whittingham in 1980 (14). In this study, they considered handling media, response of the 8-cell embryo to in vitro culture, the most suitable developmental stage for freezing, cooling and warming protocols and in vivo transfer of blastocysts. These two investigators made several observations. The in vivo viability of the rat embryos was lower after freezing and thawing than those reported for the mouse. The survival after transfer to a pseudopregnant recipient was the same for control groups and those embryos frozen slowly to -80°C and

warmed slowly. Embryos frozen slowly to -40°C before rapid cooling to -196°C and subsequent rapid warming gave a lower survival rate as measured by embryo transfer. They did not suggest optimal rates of cooling and warming for the rat blastocyst.

Once again, there appeared to be a need for better in vitro culture techniques for the rat embryo. Wood and Whittingham also suggested the need for a method of increasing egg yield from individual females (14).

Rat embryos can be successfully frozen, thawed and then transferred to pseudopregnant females (13,14). The need for a better understanding of nutritional requirements for in vitro cultivation appears to be a major obstacle in the development of optimal cooling and warming protocols for the rat embryo. In vivo transfer of frozen and then thawed rat embryos yields lower survival rates than those reported for the mouse. This technique also needs improvement, especially with respect to the induction of and recognition of pseudopregnancy.

A. General Principles of Freezing

Injury to cells during cooling to and warming from subzero temperatures is related to the fate of their intracellular water during cooling (66). Mazur (49) gives an excellent review of the cellular events occurring during freezing and thawing. As the temperature decreases, ice forms in the extracellular solution only, as the plasma membrane acts as a barrier to ice crystal growth (50). The intracellular water supercools. This supercooled water flows out of the cell and freezes externally in response to the difference in chemical potential across the membrane. The rate of cooling determines the subsequent

physical events. If the cooling rate is sufficiently slow, the cell will lose water at a rate which will maintain osmotic equilibrium between the intracellular and extracellular solutions. Because of this dehydration, the probability of formation of damaging amounts of intracellular ice will be reduced (49). If the cooling rate is rapid the cell will be unable to lose water fast enough to maintain equilibrium and the intracellular water will become increasingly supercooled. When the nucleation temperature of the cytoplasm is reached, intracellular ice crystals will form.

The presence of large amounts of intracellular ice is highly correlated with cell death on thawing (66,67). Ice crystal size is inversely related to cooling rate; therefore, these intracellular crystals will be small in rapidly cooled cells (66). These small crystals have a higher surface energy than larger ones and on thawing some of these crystals will grow at the expense of others if warming is slow. This is known as recrystallization (49). If the warming rate is rapid, the probability of recrystallization will be reduced.

Cryoprotectants do not protect against intracellular freezing only against slow freezing injury. Mammalian embryos survive cooling at very slow rates, therefore, the addition of an effective cryoprotectant is a necessity for survival.

B. Cryoprotective Additives

The main causes of injury during slow cooling, to subzero temperatures then, are the results of osmotic stresses caused by the

removal of water from cells in response to increased osmotic pressure extracellularly as water freezes in the suspending medium (68).

Artificial cryoprotection was first described by Polge, Smith and Parkes in 1948. They showed that glycerol afforded protection against freezing injury in red blood cells and bull sperm (67). In 1959, Lovelock and Bishop discovered the cryoprotective ability of Me_2SO (69). This substance had all the properties required of a solute to render protection to living cells against osmotic stress during freezing. These properties include: very low toxicity, low molecular weight, high solubility in aqueous electrolyte solutions and the ability to permeate living cells (69). In this study they showed that less Me_2SO was required for complete protection of red blood cells and it permeated more quickly than glycerol. They concluded that the ability of Me_2SO to protect against freezing injury was due to its' ability to decrease the extent to which salts concentrate when water is converted to ice.

Since these initial reports, a variety of compounds have been found which afford cryoprotection. Only two protect mammalian embryos, glycerol and Me_2SO and of these, the latter has been the most widely used (70).

Glycerol and Me_2SO are thought to prevent potential deleterious exposure of cells to elevated concentrations of electrolytes by their colligative action, in decreasing the amount of ice formed at any given subzero temperature (18,68).

The survival of mammalian embryos exposed to subzero temperatures is dependent on two major factors; the presence of protective solute and the rate of cooling and warming (17,29).

Not only the concentration of the cryoprotectant but the rate at which it is added to the embryos and the method and rate of dilution are important to survival. The length and temperature of exposure of the additive to the embryos is also a very important consideration (70).

All investigators have found that the optimal concentration of Me_2SO for survival of mammalian embryos ranges between 1 and 2 molar (70). The rat embryo has been successfully frozen at concentrations of 1.5 M (13,14). Very high concentrations of Me_2SO are detrimental to living cells (70). During the cooling process embryos in 1 M Me_2SO are exposed to 10 times that amount due to the concentration of solutes as ice forms (71). These concentrations, however, are not lethal, as viable young are obtained when embryos are thawed and transferred to recipient females.

As was mentioned before, the rate of addition of cryoprotective agent is extremely important. Many investigators report best results when Me_2SO is added in a stepwise fashion (13,14,17,72). Other researchers have obtained equally high survival rates when Me_2SO is added in a single step (29,73).

When a cell is placed into a hyperosmotic solution of a permeating solute such as Me_2SO, the cell quickly shrinks by losing water to restore the osmotic equilibrium across its membrane (70). Once osmotic equilibrium has been reestablished, the cells slowly increase in volume as Me_2SO enters with a concomitant reentry of water (73). This progressive entry of solute and water continues until chemical potential equilibrium across the membrane has been restored. The time-dependent permeation of solute into a cell is a function of the initial solute concentration and the temperature of exposure. The greater the concentration and the higher the temperature, the faster the solute

permeates (70). The rate of permeation is also influenced by the permeability characteristics of embryos from various species and at various stages of development (74).

The permeability of a cell to a specific solute such as Me_2SO is described by it's permeability coefficient, temperature coefficient and the reflexion coefficient (75). Briefly, the permeability coefficient is a measure of how fast a substance can pass through a cell membrane. The surface area available for this would also play a role in permeation and could explain some of the differences in permeability seen in various stages of the mouse embryo (76). The temperature coefficient of cryoprotectant permeability in mouse and bovine embryos is 2.5 which means that a 10°C change in temperature alters the permeability coefficient by a factor of 2.5 (77). Because of this evidence, Schneider suggests that exposure to cryoprotectants should be done at room temperature (78). He suggests a decreased temperature would unnecessarily prolong the time required for permeation of the protective solute.

Studies done with 8-cell mouse embryos exposed to various concentration of Me_2SO at 0°C exhibit changes in volume which are consistent with the osmotic responses of cells exposed to hyperosmotic solutions of permeating solutes (73).

Because Me_2SO is more permeable than glycerol, studies have shown that the time required for equilibration prior to cooling is not as critical as the times required when using glycerol (70). Most investigators add Me_2SO at 0°C and allow approximately 15-20 minutes for osmotic equilibration. Other workers have added the protective solute at room temperature (14,72) with no change in viability. Mouse embryos do

not appear to be sensitive to the time of exposure to the cryoprotectant. Rabbit embryos, on the other hand, survive better if the exposure time is at least 30 minutes (79).

Some investigators argue that permeation is not required for protection against slow freezing injury in embryos (73). They found no difference in survival when 8-cell mouse embryos were equilibrated for 5 minutes to 60 minutes in 1 M Me_2SO prior to freezing and thawing. Their observations of changes in volume of the 8-cell embryos exposed to this concentration of Me_2SO showed no re-expansion of the blastomeres until after 6 minutes of exposure. They also concluded that the zona is very permeable to the protective solute Me_2SO.

The protective nature and permeability of Me_2SO is not fully understood but it is the most widely used cryoprotectant for embryos. The effect of temperature, time and rate of permeation of cryoprotective solute in the rat embryo has not been determined. Most studies in the rat, to date, follow the methods developed for mouse embryos as these are similar in size and cell volume (74,80).

The dilution of the protective additive is just as important as its addition. On thawing, the embryo is exposed to decreasing concentrations of solutes as the ice melts. This, as does the cooling process, subjects the embryos to large changes in volume and changing osmotic gradients.

Once thawed, the suspending medium is at the same osmolarity as it was prior to freezing. During dilution, the response of the embryos to a decreased solute concentration depends on the amount of cryoprotectant which permeated them prior to freezing and on their relative permeability to solutes and water (70). Since the blastomeres are much more permeable to water, the embryos respond to dilution by rapidly taking up water and

swelling. As with the addition of cryoprotective agent, the rate and degree of response is also influenced by temperature (74). Embryos can withstand an increase in volume only by a factor of 2 without losing their developmental capacity (78). Theoretically, if mouse embryos in a 1.5 M glycerol solution are equilibrated into isotonic medium in a single step the osmotic gradients will force the embryo to swell to 5 or 6 times their isotonic volume (78). To avoid this damaging osmotic shock, the cryoprotective additive is diluted out in a stepwise manner generally at 0°C (29,70,74). Recently, there have been reports of increased survival when the temperature of dilution is 22°C and the dilution steps are smaller (70,74).

Another method of limiting the amount of swelling during dilution is by including sucrose, which is a non-permeating substance, in the medium (78). Sucrose maintains a constant external osmolarity and has sufficient osmotic pressure to prevent injurious cell swelling during the efflux of intracellular cryoprotectant (70). Me_2SO moves out of the blastomeres and water moves into the cells in response to concentration differences in the extracellular and intracellular solution. Embryos regain their normal volume once transferred to a sucrose-free isotonic medium. The time allowed for the efflux of additive is generally between 20 and 30 minutes prior to dilution of the sucrose with isotonic medium (70).

C. Rate of Cooling

The events which occur in the suspending medium and the embryo when exposed to decreasing temperatures were discussed previously in this review (page 20). A more detailed look at the effects of cooling and

warming rates in mammalian embryos will now be considered. Control of the cooling and warming rates determines the ultimate fate of intracellular water during cryopreservation. If cooling is slow, water will move out of the cells and freeze extracellularly. If however, the rate of cooling is too rapid, the cytoplasm will not have sufficient time to dehydrate, will supercool and eventually freeze. The formation of intracellular ice is thought to lead to injury (67).

In the past thirty years, the development and refinement of the cryomicroscope has allowed observation of cells during cooling and warming (18). Quantitative data on volumetric changes during freezing and the formation of intracellular ice have since been obtained for many cell types. Viability assays must be paralleled with the observations obtained from this technique in order to interpret observations from the cryomicroscopic information. Embryos can now be recovered from the low temperature stage and then viability analyzed using in vitro culture (18).

When embryos are cooled below 0°C they only begin to respond osmotically after ice initially forms (70). If the temperature continues to decrease, the suspending medium and embryos will supercool until their respective ice nucleation temperatures are reached or before, if other ice nucleators are present. The homogeneous nucleation temperature for mouse embryo cytoplasm in 1 M Me_2SO is -40°C (81). Before ice formation can begin under any condition, nucleation is required. There are two types of nucleation. The first, homogeneous nucleation, occurs at or below -36°C depending on the composition of the solution. It involves the spontaneous aggregation of water molecules to form ice nuclei (82).

The second, heterogeneous nucleation, occurs below the freezing point of a solution providing ice nucleators are present (83).

If ice formation in the extracellular solution is not induced above -10°C, the probability of damaging amounts of intracellular ice may result (82). When ice forms the release of the latent heat of fusion causes the sample temperature to rebound close to the freezing point of the solution. The cooling rate of the sample will then exceed the optimal rate as the sample temperature equilibrates with the cooling bath temperature (78). This would increase the probability of formation of damaging amounts of intracellular ice.

To overcome these deleterious events, samples are held in a constant temperature bath or seeding bath a few degrees below their freezing point. Several minutes are allowed for the samples to supercool and ice crystal formation is induced by either tapping the container sharply, touching the solution surface with a pipette tip containing frozen medium or by touching the container side near the fluid meniscus with a cold metal rod (70,78). Crystallization will continue until the chemical potential of water in the unfrozen fraction is in equilibrium with the chemical potential of ice (78).

Once ice formation is induced, time is needed for the latent heat of fusion to be released, for thermal equilibrium between the sample and the seeding bath temperature to be restored and to allow the embryos to respond osmotically to the changing extracellular solute concentrations (78). The cytoplasm is still presumed to be supercooled after seeding as the plasma membrane acts as a barrier to ice crystal growth (50).

It has been established, that during freezing and thawing embryos undergo many changes in volume as a consequence of osmotic forces.

During the addition and dilution of cryoprotectant the osmotic gradients are due to the extracellular presence or absence of that cryoprotectant. During freezing the osmotic gradients are related to temperature changes (78).

The major cause of death to embryos frozen and thawed rapidly, is the formation of intracellular ice during cooling (74). The damage is manifested only on warming and may be related to the recrystallization of ice (49), or to the osmotic stresses imposed once the ice melts (51). The cooling rate must be slow enough to allow sufficient dehydration of the cytoplasm in response to increasing solute concentration as ice forms in the extracellular solution. The movement of water is dependent on the volume of the cell, its surface area, its permeability to water and the temperature coefficient of that permeability (74). The total amount of dehydration also depends on the temperature when intracellular ice forms.

Because embryos have a comparatively low permeability to water and a low surface area to volume ratio (84), a slow cooling rate is prescribed. The first mammalian embryos frozen successfully in 1972 by Whittingham et al. (29) and Wilmut (17), were cooled at a rate based on calculations made by Mazur for sea urchin eggs which are comparable in size to the rat and mouse embryos (74).

Leibo studied the events occurring in mouse ova during slow and rapid cooling using cryomicroscopic observations and volume measurements (81). His observations indicated a critical event which occurred between -35°C and -45°C in ova cooled in solutions containing 1 M Me_2SO. Ova cooled rapidly, 3°C or 30°C/min, froze intracellularly at about -40°C indicated by a sudden darkening of the cytoplasm, referred to as flashing. If samples were cooled at 1°C/min, no flashing occurred, even

to temperatures as low as -135°C. None of the ova froze intracellularly above -30°C and all of those that did freeze, did so above -60°C. Leibo concluded that intracellular ice nucleation occurred at about -45°C. His observations also revealed that ova cooled at 1°C/min diminished in volume considerably between 0°C and -135°C, where those cooled at faster rates did not show a significant change in volume. He showed that survival of ova depended not only on the rate of cooling but also on the minimum subzero temperature to which they are cooled slowly.

Cryomicroscopic observations show the absence of intracellular ice in embryos cooled slowly to -40°C (18). At -40°C, 74% of the extracellular solution had crystallized and the concentration of solute in the remaining liquid was four times greater than in a 1.5 M Me_2SO solution. If the samples were rapidly cooled from -40°C to -196°C, Rall observed no further crystallization. The residual liquid vitrifies or forms a glassy state without a change in its composition (18). Embryos frozen slowly to -80°C before rapid cooling to -196°C have dehydrated to equilibrium prior to reaching their nucleation temperature and theoretically have a zero probability of undergoing intracellular freezing (49).

Controlled cooling to an intermediate subzero temperature either -40°C or -80°C, can be safely followed by rapid cooling (-250°C/min) to liquid nitrogen. Cryomicroscopic observations show that during rapid cooling to -196°C there is no change in the embryo indicating the absence of visible intracellular ice (18). One change which has been observed is the formation of a series of fracture planes which suddenly appear in the extracellular ice matrix. These are sometimes seen to pass through a

zona and occasionally the cytoplasm of an embryo resulting in a cracked zona and damaged blastomeres on thawing (18).

D. Length of Storage

Theoretically, embryos can be stored indefinitely in liquid nitrogen at -196°C. Below -120°C there are no thermally driven reactions in aqueous solutions (78). The only reaction possible at -196°C is DNA breakage caused by background radiation (85). Some protection is afforded against ionizing radiation by Me_2SO (86). Experiments using frozen 8-cell mouse embryos exposed to the equivalent of 2000 years of background radiation did not produce any detectable genetic changes or reduce viability (85). Normal levels of background radiation then, would not be a hazard to the long term storage of mammalian embryos.

To date, 8-cell mouse embryos have been stored in liquid nitrogen for varying periods up to 11 years with no evidence of deterioration of survival with time. F1 hybrids survive best, and stocks of mutant mice strains have been reestablished (12).

E. Rate of Warming

The survival of mammalian embryos is as dependent on the rate of warming as it is on the rate of cooling (70). The first successful reports on the cryopreservation of mouse embryos noted the extreme sensitivity of slowly cooled embryos to the rate of thawing (17,29). In more recent work, investigators have shown that the rate of warming is dependent on the cooling regime used (74,78). If slow cooling is

controlled to -60°C or below, optimal survival is obtained when embryos are thawed slowly (4-25°C/min) (87). If however, the slow cooling is terminated between -35 and -40°C by direct transfer to liquid nitrogen, mouse 8-cell embryos and blastocysts survive rapid warming (200°C/min) (87).

Rapid warming of slowly cooled embryos to temperature of -60°C or below results in injury (73). Me_2SO solutions have very low eutectic points, or they start to melt during warming at temperatures as low as -60°C to -70°C. Embryos frozen in this solution would be exposed to large and rapid changes in solute concentration between -70°C and 0°C when the solution is warmed rapidly. If warming is slow, the solutes would be diluted gradually as solvent water returned to the system during melting. This suggests that the damage to embryos in the rapidly warmed samples is a result of osmotic shock (73).

Survival of embryos frozen slowly to higher subzero temperatures (-40°C) prior to rapid cooling to -196°C is dependent on rapid warming rates (30). These embryos contain intracellular ice and if thawing was slow, these ice crystals may undergo recrystallization and cause death (18,88).

Cryomicroscopic observations have enabled scientists to better understand the phenomenon of thawing. Eight-cell mouse embryos were cooled slowly to -40°C and then rapidly to -196°C and subsequently thawed at different rates under the cryomicroscope (18,88). If these were then thawed rapidly at 110°C/min or faster there was no visible change in the embryos. Extracellular ice melted above -40°C, and these embryos divided in culture. Rall *et al*. concluded that at this warming rate sufficient time was not allowed for formation or growth of ice nuclei (88). If, on

the other hand, these embryos were warmed slowly, intracellular ice formation was observed at -90°C indicated by a sudden darkening of the cytoplasm. Injury only occurred in embryos warmed slowly to -65°C or above. Two other events occurred if slow warming continued. A second flashing at -55°C, migratory recrystallization, and the melting of extracellular ice at -40°C (18,88). From this work, Rall et al. concluded that slow warming injury in embryos cooled slowly to -40°C was not due to the formation, recrystallization or melting of intracellular ice (18). This recent evidence contradicts both theories of slow warming injury, those of osmotic events when ice melts and migratory recrystallization. Rall concluded that injury occurs before both of these events (18).

Rat embryos have been successfully frozen to both -80°C and slowly warmed and to -40°C and rapidly warmed (14). This study suggested that the blastocysts survived the former protocol slightly better than the latter. Wood and Whittingham did observe that on morphological examination just after thawing more embryos cooled slowly to -40°C appeared normal however. Rates of in vivo survival were low in both cases.

F. Stage of Embryo Development

Another consideration for optimal cryopreservation of mammalian embryos is the best preimplantation stage of development for manipulation, cryopreservation and viability assays. Since all preimplantation stages remain free and unattached in the female reproductive tract and are relatively easy to collect (74), other

considerations include, which stages respond best to *in vitro* assays and which stage can be most successfully and easily transferred to a foster recipient.

In 1972, Wilmut reported no difference in survival between mouse 8-cell and blastocysts when frozen to -196°C and thawed (17). Whittingham *et al*. froze all preimplantation stages of the mouse embryo in 1972 and reported better survival for the 1- and 2-cell as tested by *in vitro* cultivation (29). To date the mouse 8-cell is most widely used in cryopreservation techniques and for embryo banking (74).

In the rat, fewer studies have been carried out. Whittingham, in 1975, froze 2-, 4- and 8-cell rat embryos (13). Since there is no reported development beyond the 4-cell stage (27), the 8-cell responded best in culture (13). In 1980, Wood and Whittingham chose to freeze the rat blastocyst only in their study. Their reasons were numerous. There is no development beyond the 4-cell stage and 2- and 4-cell embryos must be transferred to the oviduct for optimal survival. This technique is more difficult in the rat as the oviduct is extremely vascular (32), therefore, transfer of the 8-cell embryo or blastocyst into the uterus is simpler. They did preliminary work with 2-, 4- and 8-cell embryos and found *in vivo* survival low irrespective of technique. These investigators were not satisfied with the *in vitro* viability of the 8-cell rat embryos (14).

Again, these investigators feel that viability assays are inadequate in the rat embryo and before optimal cryopreservation techniques can be developed, *in vitro* culture and *in vivo* transplantation techniques must be studied.

Review of Viability Assays

The importance of reliable viability assays to determine optimal cryopreservation protocols is crucial. Both *in vitro* culture for immediate assay prior to further experimentation and *in vivo* assays for ultimate viability assessment and reestablishment of various genetic strains must be better understood before the rat embryos can be successfully cryopreserved.

A. Fluorescein Diacetate Assay

The use of dyes as a test of viability for the mammalian embryo has not been widely employed. The use of a fluorescent dye has been used in a few studies for assessment of initial viability of frozen-thawed embryos (89,90). This assay was first described by Rotman and Papermaster in 1966 (91). Fluorescein diacetate (FDA), is a nonpolar compound and readily passes into the cell where it is hydrolysed by esterases to form fluorescein. This product is polar and cannot readily cross the plasma membrane and therefore accumulates intracellularly (91). This assay measures both esterase enzyme activity and membrane integrity (92).

Incubation times required for this assay are short and the embryos are observed under ultra violet (UV) light, using a fluorescent microscope. Investigators which have used this technique report a high correlation (0.96) between the ability of an embryo to fluoresce and subsequent ability to develop in culture (89).

In a more recent investigation, Mohr and Trounsen (92) studied the effect of FDA and UV exposure on all stages of preimplantation mouse embryos. They found no effect of FDA or UV light on the ability of the embryos to continue development in vitro or in vivo.

This technique may be an effective initial test of viability post-thaw prior to further manipulations in the mammalian embryo.

B. In Vitro Embryo Culture

(i) Development of Mammalian Embryo Culture Techniques

"The first essential step in the analysis of early mammalian development is the establishment of suitable techniques for culturing embryos outside the maternal environment." (62)

The in vitro cultivation of mammalian embryos was begun in 1893 (37). In the past 15 to 25 years a great deal of knowledge has been gained about the nutritional requirements of the preimplantation stages of mammalian embryos. The simulation of the maternal environment has proven to be a formidable task. There are differences between species as well as differences between developmental stages in a given species (62).

The development of in vitro culture methods has been carried out using the mouse and rabbit embryo primarily. Briefly, Hammond reported the successful culture of 8-cell mouse embryos to blastocyst using saline with glucose and thin egg white (41). He observed no division of 2-cell embryos. In 1956, Whitten reported successful cultivation of mouse 8-cell embryos to blastocyst substituting BSA, for egg white (42). The pH of this medium was more stable and the addition of BSA altered the physical properties of the medium as embryos did not divide without this

component. BSA also facilitated the manipulation and handling of the embryos (26). In 1957, Whitten discovered the benefits of lactate on the development of late 2-cell mouse embryos to blastocysts (26). McLaren and Biggers, one year later, obtained live mice from 8-cell embryos cultured to blastocysts and then transferred to recipient females (16). There was a definite developmental blockage between the cleavage of the 2-cell mouse embryo and the 4-cell stage (16,41,42). Blockage was described to be due to a metabolic deficiency in the glycolytic pathway (93). Two-cell mouse embryos would divide in the lumen of oviducts (16), therefore, it was obvious embryos at this stage had special requirements not yet discovered.

In 1963, Brinster described the medium most used today for cultivation of mouse 2-cell embryos to blastocysts (15). Again in 1965, Biggers and Brinster demonstrated that the inclusion of pyruvate and lactate in the culture medium gave even better results. Few changes have been made in the techniques for culture of mouse embryos to date.

Development of culture techniques for the rat embryo have not been as successful. As discussed above in the overview of embryo research, Defrise reported on the normal and abnormal morphological criteria of various stages of the preimplantation rat embryo (40). Although his techniques were crude, he noted that these embryos were very sensitive to changes in temperature, osmotic pressure and pH of the holding medium. In 1969, Folstad, Bennet and Dorfman cultured 2-, 4- and 8-cell rat embryos testing various media, energy and nitrogen sources and several tissue culture systems (28). They reported success (90%) when culturing 8-cell rat embryos to the blastocyst stage using Brinsters method described in 1963 (15). Sodium pyruvate and lactate were used as the

energy sources and BSA as the nitrogen source. Another study was published in 1969 where rat embryos were cultured within the oviduct lumen (94). These investigators did not observe more than one division in culture using conventional techniques, and few developed past the 4-cell stage even in the oviduct. In 1974, Mayer and Fritz reported successful culture of all preimplantation stages of the rat embryo through at least one cleavage stage with the exception of the 4-cell (27). They obtained an 80% success rate when culturing the 8-cell rat embryo to the blastocyst stage. The culture method these investigators employed was that described by Biggers, Whitten and Whittingham (37). These workers substituted FCS for BSA and observed that this initiated hatching of the blastocyst from the zona pellucida.

In these studies, the investigators observed a blockage in development between the 4- and 8-cell rat embryo which may be similar to that observed in the 2-cell mouse embryo (27).

Further work in this area of in vitro culture of rat embryos has involved the use of this technique as a viability assay after cryopreservation (13,14). Both studies indicated the need for improved culture methods for the rat embryo and a better knowledge of the nutritional requirements for the preimplantation stages of development.

(ii) Technical Considerations

The most important factors affecting successful cultivation of mammalian embryos are; the culture medium, the culture environment and sterility.

The nutritional requirements vary from species to species as well as between stages of development in one species (24,62). A simple

chemically defined medium is sufficient to support development, provided the proper energy and nitrogen sources are included (62). Embryos are far more sensitive to variations in medium than are other mammalian cells and tissues, for example, they are unable to tolerate wide variations in pyruvate and oxygen concentration or the presence of metallic ions (62). The inclusion of pyruvate and lactate as energy sources was realized by 1965 (23). Both are thought to eliminate stress of the metabolic processes as the early embryo develops. The action of pyruvate is twofold, as it acts as an energy source for metabolism and for biosynthetic processes occurring in early development (62). Glucose is also a requirement for mammalian embryo development but cleavage is supported best in the presence of all three energy sources (24).

The need for a nitrogen source was also recognized early in the development of culture techniques for mice and rabbits (26,42). Bovine serum albumin is widely used as this nitrogen source although some investigators prefer FCS (14,27). Mayer and Fritz believe that FCS enhances hatching of the blastocyst from the zona in vitro (27).

The role of BSA or FCS may be to stabilize the plasma membrane and to reduce leakage of endogenous amino acids into the culture medium (95). These protiens may also aid in removing toxic metal ions present in the culture medium (24), as well as supplement the endogenous amino acid pool.

The importance of strict control over pH was first recognized by Defrise in 1933 (40). In 1956, Whitten reported that 8-cell mouse embryos would cleave in medium with a pH of 6.9 to 7.7 (42). The hydrogen ion concentration in bicarbonate-buffered medium is controlled by the level of bicarbonate in the medium and the amount of CO_2 in the

atmosphere (62). Since bicarbonate-buffered medium is most commonly used for mammalian embryos, the atmosphere of the culture chamber usually contains 5% CO_2 in air at a temperature of 37°C (24). The pH is generally maintained at 7.4 although this area of embryo culture has not been extensively researched (24).

Regulation of humidity is also very important. If the culture chamber is not saturated with water vapor, water will be extracted from the culture medium and the osmotic pressure will be elevated in the medium (24). Increased osmolarity of culture medium results in poor embryo development (95). Most culture media used for mammalian embryo culture have an ionic composition and total osmolarity similar to that of blood serum which is 0.308 mosm. Optimal osmolarity for development of mouse 2-cell embryos to blastocyst in BMOC-3 is 0.276 mosm as reported by Brinster (23). This is slightly hypo-osmolar but the reason for this is not known (24).

There are several techniques for mammalian embryo culture and a good review is given by Brinster (24). Only two methods will be discussed in this review. The first is the method most commonly used today and was developed by Brinster in 1963 (15). It involves placing a microdroplet of culture medium beneath a layer of sterile equilibrated paraffin oil. Embryos are transferred to these droplets of medium. The second method was first described by Hammond (41). A small amount of medium is placed in a serological tube and embryos are placed in the bottom of the tube. This method was used in some of the first successful culture experiments for mouse embryos (26,42). In both of these methods the atmosphere, temperature and humidity are strictly controlled (24). Brinster reports

a greater loss of embryos on recovery using the latter method and most investigators employ the former.

(iii) Criteria for Development

One of the most important considerations for utilization of in vitro cultivation as a measure of viability is to develop a strict criteria for development (62). Only embryos whose morphological appearance is normal should be used for any assay of viability. The recognition of normal and abnormal cleavage divisions is a learned technique and knowledge of normal and abnormal appearance of the embryos is absolutely necessary (37,62). Most investigators use quantal (all or none) responses to measure success of in vitro culture (96). These may include, development past the 8-cell stage, or formation of a blastocoele. "It is necessary to employ a characteristic which is clear cut visually to minimize bias arising from subjective judgements and one which allows a sufficient test of normal morphological development." (96)

Best results are obtained if experimental groups include more than 10 embryos for statistical consideration (62).

In the mouse, there is a high correlation between the ability of embryos to develop in culture and their ability to develop into normal live offspring following subsequent transfer to a foster mother (74).

C. In Vivo Transplantation

The transfer of a donor mammalian embryo to a pseudopregnant recipient female is the best test of viability. This technique is extremely complex and many variables determine success. The ability to

recognize normal embryos for transfer is very important as was mentioned earlier in this review, (page 16). Only morphologically normal embryos should be transferred. The experience of the operators is very important and this is definitely a consideration at the beginning of experiments.

The transfer of embryos requires a knowledge of the estrus cycle of the female rat, the normal development of preimplantation stages of the embryos, induction of pseudopregnancy and the ability to recognize this state, as well as transplantation experience.

The first report of successful transfer of rat embryos was in 1933 by Nicholas (39). He transferred hooded 2- and 4-cell embryos which are dominant for black eyes, into albino recipient females. Nicholas reported 60% of embryos transferred developed into normal live offspring.

In 1960, Dickman and Noyes experimented with the synchronization of rat embryos and the recipient's day of pseudopregnancy (34). They determined that survival of the transferred embryos was dependent on the age of the embryo, stage of development of the recipient's uterus and a particular relationship which existed between the two. They also showed that the rat embryo implants on day 5 of pregnancy. These investigators reported on the difficulty of transfer to the oviduct, the importance of synchronization, especially for older stages (blastocyst), and described the process of implantation (33,34).

The use of <u>in vivo</u> transfer of mammalian embryos as an assay of viability for cryopreservation is widely employed (14,29,97-99). The success of this technique is much higher for the mouse than the rat. The major problem in the rat is that of low survival rates even in control experiments.

(i) Induction of Pseudopregnancy

Pseudopregnancy is a false state of pregnancy. It can be induced using one of two methods, mechanical stimulation of the vagina and cervix (100), or mating the female with a vasectomized male (58). Induction of pseudopregnancy by either of these methods is essential in rats to transform the cyclic corpora lutea into functional ones after ovulation (31,32). Both of these methods require the use of mature females and careful observation of the estrus cycle, usually by daily vaginal lavage (58,100).

Mechanical stimulation is achieved by inserting a simple metal probe attached to a vibrator on two days in a row when the vaginal smear shows proestrus and estrus (100). This method is claimed to be 100% effective.

The other method, which is more popular in the literature, is mating recipient females with a vasectomized males. Usually males are chosen which have a genetic marker, such as dominant black eyes. The female is mated on the night the vaginal lavage shows proestrus. The female is considered to be in day 1 of pseudopregnancy the morning following mating.

The normality of the embryos transferred is very important (32). To eliminate some of the variables affecting success, only embryos which appear absolutely normal and are in the desired stage of development should be transferred (101). The time-scale of development for the rat embryo has been discussed earlier in this review (page 15). The recognition of normal and abnormal cleavage has also been mentioned (page 16) and will not be discussed here.

(ii) Technical Considerations

The technical considerations for this technique are many. The handling medium and operating room conditions are important. Synchronization of donor embryos and recipient are of utmost importance as are the site of transfer, number of eggs transferred and amount of experience the operation has achieved (32). There is a large variation in the ability of early embryos, up to the 8-cell stage, to continue development when transferred to the uterus (31). This is related to the stage of the uterus when embryos normally enter under natural pregnancy conditions.

Much work has been done on the synchronization of age of donor embryo in relation to stage of pseudopregnancy. Dickman and Noyes in 1960 (34), reported that rat embryos would not implant if younger than 3 and older than 5 days of age. The endometrial environment compatible with survival of transferred ova persisted from the third to fifth day of pregnancy (34). One of their most important observations was that only ova of the same age or one day older than the corresponding stage of the uterine development would implant once the recipient reached the fifth day of pregnancy. With these observations they concluded that on the fifth day of pregnancy the uterine environment changes and is only receptive to embryos in the correct developmental stage for implantation. This work was done using albino donor embryos and already pregnant hooded (black eyes) recipients.

In later work, these investigators used pseudopregnant female rats as recipients (33). They obtained best results with transfer of day 4 (8-cell) embryos or day 5 blastocysts into day 4 pseudopregnant females. Synchronous transfer of blastocysts resulted in slightly lower survival.

Until day 5 both the ovum and the endometrium develop independently. On day 5, however, the uterine environment suddenly changes and becomes detrimental to underdeveloped ova and stimulates developed or slightly over developed ova in such a way that they attach themselves and elicit the decidual reaction. Beyond day 5 they found that both the embryo and the uterus lose their ability to participate in implantation. They also suggested the use of F1 hybrids for the best results.

The site of transfer is also important. Generally the site of transfer is governed by the developmental stage of the embryo (32). In mice, early stages of development, fertilized ova, 1- and 2-cell embryos, can be transferred to the ovarian bursa or oviduct with success (58,99). This technique is far more difficult in the rat as the bursal membrane is very vascular and trauma caused to the reproductive tract is detrimental to implantation (32). Four day embryos should be transferred to the uterus on day 4, and 5 day embryos on days 4 or 5 respectively in both mice and rats (33).

The handling medium for collection, manipulation and transfer also effects the success of <u>in vivo</u> transfer of rat embryos (32). Whittingham found that wide and rapid temperature fluctuations were detrimental to embryo survival. It was best if medium was maintained at a constant room temperature than if moved in and out of a culture chamber at 37°C for holding times up to 3 hours (31). Wood and Whittingham expressed a need to study the effect of the pH of the handling medium on embryo survival when carrying out transplant experiments (14).

The importance of sterility is also evident as the early luteal phase uterus is particularly susceptible to infection (32).

Despite these problems, the use of embryo transfer as a viability assay after *in vitro* manipulation is a valuable and useful tool. In mice, this technique is the one most often employed and is obviously the most stringent viability assay. In the rat, however, success has not been as great, and a better understanding of the reproductive system and of embryo development and viability *in vitro* is needed.

III

MATERIALS AND METHODS

Materials

Pregnant mare serum gonadotrophin, human chorionic gonadotrophin, fluorescein diacetate and bovine serum albumin were obtained from Sigma Chemical Company. Dulbecco's phosphate buffered saline, Brinster's medium, and phenol red were obtained from Gibco Laboratories. Dimethyl sulfoxide, sodium lactate, sodium pyruvate and methyl blue dye were obtained from Fisher Scientific. Penicillin-streptomycin was obtained from Whittaker M. A. Bioproducts. Sodium Pentobarbital was obtained from M.T.C. Pharmaceuticals.

Animals and Care

The spontaneously diabetic Bio-Breeding (BB) Worcester rats were supplied for this research by the Animal Resources Division, Health Protection Branch, Health and Welfare Canada. Immature female BB rats were received at 25 to 30 days of age. Mature male Sprague Dawley breeders and mature female Sprague Dawley rats used as foster mothers were obtained from Biological Science Animal Services at the University of Alberta. Long Evans hooded rats, which are dominant for black eyes, were used for mating to obtain pseudopregnant female recipients. These were obtained from Blue Spruce Labs in New York.

All animals were housed in the small animal vivarium of the Surgical Medical Research Institute. They were under the care of the investigator with the assistance of the animal care staff and the advice of a veterinarian in accordance with the criteria formulated by the Canadian Council on Animal Care. Females were housed in groups of 3 and

males were individually housed in rat shoe box cages. Water and food (Wayne Lab Blocks) were provided ad libidum. Fresh bedding (Alpha Chips) was provided twice weekly. Animal room temperatures were between 20 and 22 degrees centigrade and all rooms had air filters. The light/dark cycle was 12 hours/12 hours.

Source of Embryos

A. Superovulation

Immature female BB rats (25-35 days) were induced to ovulate by administrating Pregnant Mare Serum Gonadotrophin (PMSG) and Human Chorionic Gonadotrophin (HCG) 48-59 hours apart. The hormones were dissolved in sterile saline at a concentration of 100 IU/ml. To determine the best dosages of these hormones for superovulation, the females were given varying doses of each hormone by intraperitoneal injection. PMSG was given in doses ranging from 15 to 25 IU and HCG was given in doses ranging from 10 to 20 IU. Immediately following the HCG injection each female was placed overnight with a proven breeder. The following morning females were checked for the presence of a sperm plug and placed back into their own cages. The day following mating was designated as day 1.

B. Natural Mating

To determine estrus, vaginal smears were performed in the afternoon on mature female BB rats, 6 weeks of age and older. The vaginal smears were obtained by flushing the vagina with a few drops of water in a fired pasteur pipet. The sample was placed on a microscope slide and a

drop of methyl blue dye, (20 gms/L methyl blue chloride (90%), in distilled water), was added to the sample. The slides were examined under a light microscope and the stage of the estrus cycle recorded. When a smear showed proestrus that female was placed overnight with a proven breeder. The following morning females were checked for the presence of a sperm plug and placed back into their own cages. The day following mating was designated as day 1. All females mated using this method were assumed fertilized whether or not a sperm plug was found.

Isolation of Embryos

All procedures were carried out under sterile conditions in a laminar flow hood. Embryos were obtained on day 4, (8-cell) or on day 5, (blastocysts) as follows: The female BB rats were anesthetized with sodium pentobarbital used at a concentration of 65 mg/ml and given at a dose of 0.06 ml/100 gms body weight of the rat. A simple laparotomy was performed. The oviducts were removed to obtain 8-cell embryos on day 4 and the oviducts and uteri were removed to obtain blastocysts on day 5. These were placed immediately in Modified Dulbecco's Phosphate Buffered Media (PB1) which consisted of Dulbecco's media plus sodium lactate (280 mg/100 ml), sodium pyruvate (2.75 mg/100 ml), bovine serum albumin (100 mg/100 ml), penicillin (100 IU/ml) streptomycin(100 ug/ml), and 0.4% phenol red as the pH indicator (65). This media was used for all *in vitro* manipulation, and as the suspending medium for freezing and thawing.

Once the oviducts and uteri were removed, the rats were killed by cutting the diaphragm. The oviducts and uteri which had been placed in PB1 were then processed. To obtain 8-cell embryos, the oviducts were

flushed with approximately 1 ml of PBl in a watch glass by cannulating the ostium under a dissecting microscope with a 30 gauge blunted needle. To obtain blastocysts, the ostium was cannulated and the oviducts flushed as for day 4 but the oviducts were then excised and an additional 1 ml of medium was flushed through the uterus in the caudal-distal direction. The number and stage of embryos was recorded, as well as a morphological description of each embryo. Embryos were then prepared for fresh control assays or for cryopreservation.

Controls for In Vitro and In Vivo Assays

Both fresh controls and manipulated controls were assessed on day 4 (8-cell) and day 5 (blastocysts) embryos. Fresh controls were put into culture immediately following isolation for 18 to 24 hours. Brinster's medium BMOC-3 (10), plus penicillin (100 IU/ml), streptomycin (100 ug/ml) and 0.4% phenol red was used as the culture media in all cases. The tissue culture environment was 5% CO_2 in air, at 37°C and 95% humidity. The pH of the medium was monitored closely. A fyrite gas analyser was used to check the concentration of CO_2 in the chamber. This instrument employs the "Orsat" method of volumetric analysis involving chemical absorption of a sample gas, such as CO2 (102). The reagent used to absorb CO2 is potassium hydroxide. Basically, the atmosphere in the culture chamber is pumped into the fyrite and then absorbed by the reagent by inverting the instrument. The level of the reagent corresponds to the amount of CO2 present in the air sample.

Fresh manipulated controls were assessed by first adding, then removing the cryoprotective agent Dimethyl Sulfoxide (Me_2SO). The addition and dilution of Me_2SO was carried out in a stepwise manner to

reduce osmotic stress and allow for equilibration between steps. Once the cryoprotectant was removed, the embryos were transferred to watch glasses, washed once in PBl and either assessed using fluorescein diacetate (FDA) assay or tissue culture.

Cryoprotection and Cryopreservation

All addition and dilution of the cryoprotective agent, Me_2SO was carried out at room temperature. The proceedure used for addition and dilution of Me2SO was developed in this lab for the cryopreservation of rat islets of Langerhans.

Once isolated, the embryos were washed once in PBl then transferred to a 13x100mm Kimax siliconized tube (5-10 embryos/tube), in 0.2 ml of PBl. To this was added 0.1 ml of 2 M Me_2SO in PBl and 5 minutes was allowed for equilibration. An additional 0.1 ml of 2 M Me_2SO in PBl were added and 25 min allowed for equilibration. A final 0.4 ml of 3 M Me_2SO in PBl were then added and 15 minutes was allowed for equilibration (see Figure 1). The final concentration of Me_2SO was 2 M. The tubes were gently agitated by hand following each addition. The tubes were then capped and transferred to a -7.2°C alcohol seeding bath to supercool for 5 minutes. The freezing point of a 2 M Me2SO solution in PBl is -4.5oC (74). The samples were nucleated by touching the outside of the tubes near the meniscus of the medium with a cold brass rod. The tubes were shaken gently and returned to the seeding bath for an additional 10 min to allow for the release of the latent heat of fusion.

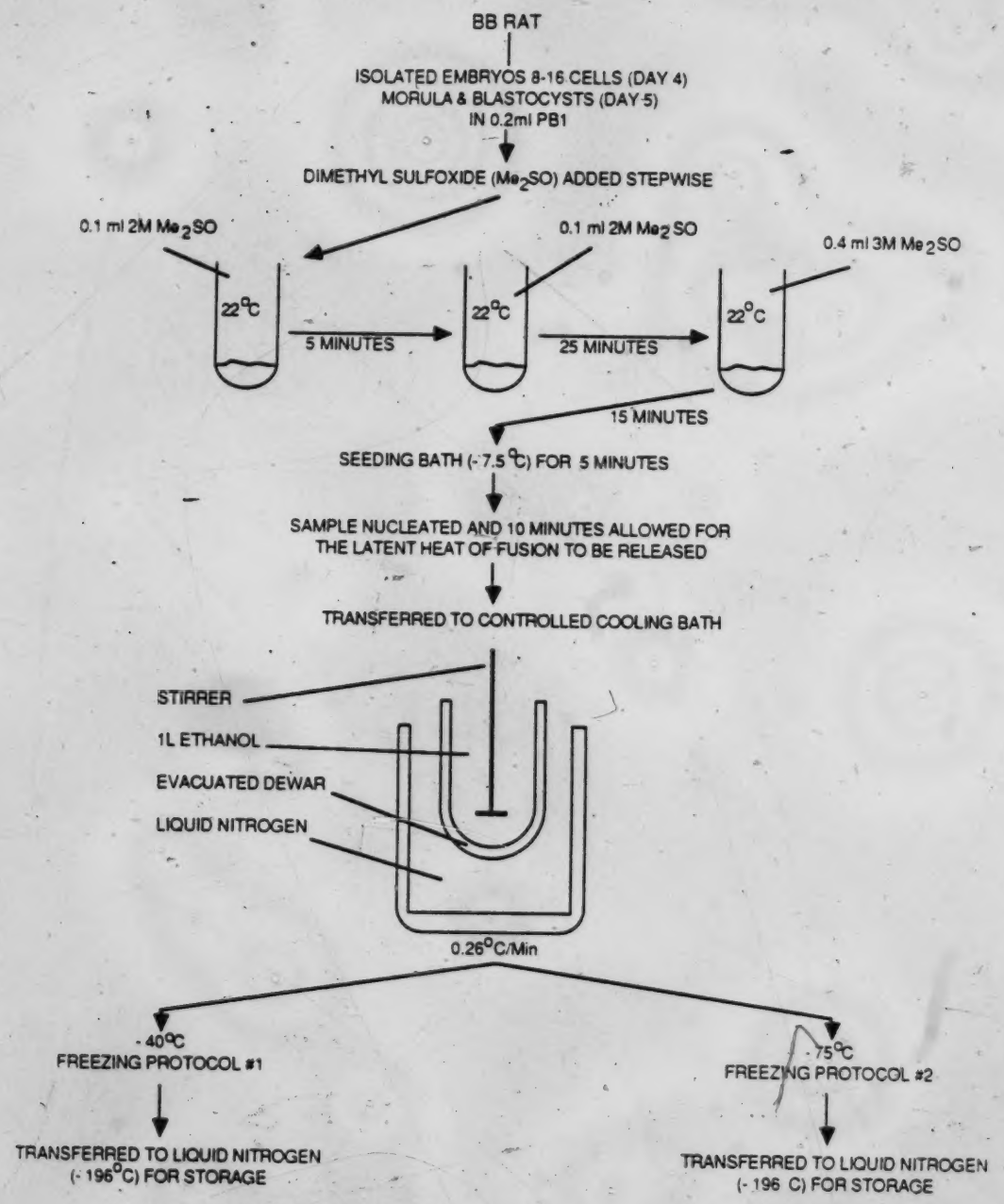

FIGURE 1: FREEZING PROTOCOL

The tubes were then transferred to an evacuated Dewar (70) filled with ethanol at -7.2oC which was surrounded by liquid nitrogen. The samples were cooled at 0.25°C/min to either -40°C or -75°C. The tubes were then plunged immediately into liquid nitrogen and stored at -196°C for varying periods of time. For assessment, the embryos cooled slowly to -40°C were thawed rapidly by gently shaking the tubes in a 37°C water bath. The thawing rate was 200°C/min. Embryos cooled slowly to -75°C were warmed slowly in room air at a rate of 18°C/minute (see Figure 2).

Stepwise dilution of the cryoprotectant was carried out at room temperature in the following manner: four 0.1 ml aliquots of PBl were added to the tubes at 5 min intervals; to 0.2 ml aliquots of PBl were then added at 5 min intervals; this was followed by adding 2 aliquots of 0.8 ml PBl and a final 3.2 mls to the tubes. Five minutes were allowed for equilibracion between each of these additions (see Figure 2). The tubes were agitated gently after each addition of PBl. After five minutes, the tubes were capped and transferred to a centrifuge. They were spun at 230 x g for 10 seconds.

Using a sterile, siliconized pipet the embryos and medium were transferred into a watchglass, counted and then transferred to isotonic PBl at room temperature. The embryos were washed once in PBl, counted and their morphological appearance recorded. They were then assessed using either <u>in vitro</u> or <u>in vivo</u> tests of viability.

<u>In Vitro Assays</u>

A. Fluorescein Diacetate Assay

FDA was made up as a 0.012 M stock solution in Acetone. This stock solution was placed in a light-insensitive bottle wrapped in foil and

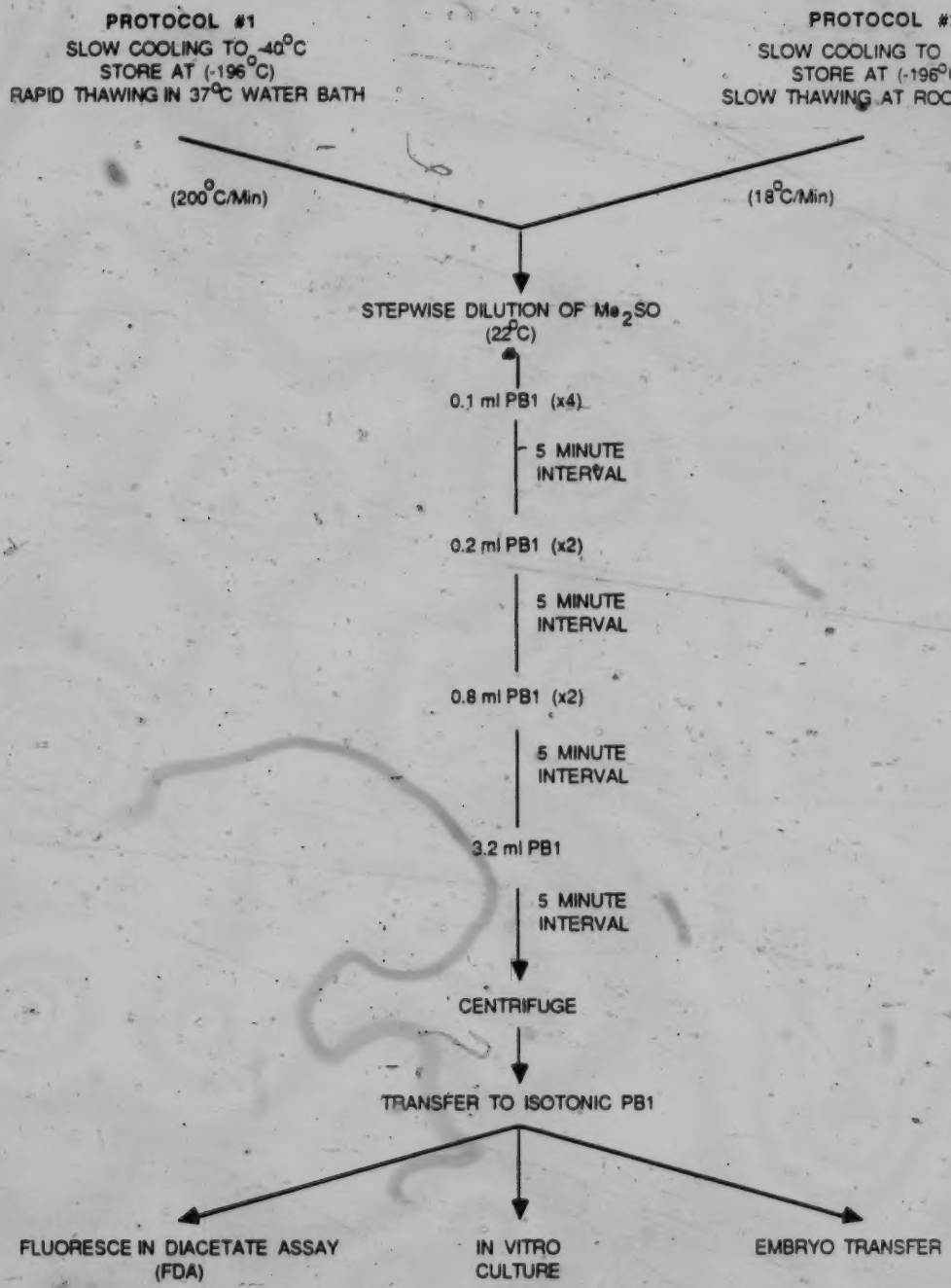

FIGURE 2: THAWING PROTOCOLS

stored at -20°C. Just prior to use the stock solution was diluted 1:100 with sterile saline and this was then diluted again 1:10 with PBl (89). Embryos were carefully transferred to a welled slide containing 3 drops of the final FDA solution. Silicone high vacuum grease was used to create a seal around the well beforehand to prevent evaporation of the medium. A slip cover was carefully placed on top of the grease and sealed. The slides were then examined under a Leitz fluorescent microscope. Embryos were identified and recorded as having either positive or negative fluorescence. Several researchers grade the degree of fluorescence (89,90,92), however, this investigator chose to record only those with all blastomeres fluorescing equally as viable. Photographic plate 1 shows a fluorescing 8-cell BB rat embryo after cryopreservation. Embryos used for this in vitro assay were not used in any other assay.

B. Embryo Culture

Initially, the traditional method for embryo culture was employed (15). Basically, embryos were placed in drops of Brinsters culture medium under sterile, equilibrated paraffin oil in Nunc micro culture plates. However, very poor results were obtained with this method and instead, batch culture was used. Approximately 0.5 ml of Brinsters medium plus penicillin-streptomycin and phenol red were placed in a 13x100mm Kimax culture tube (42). The medium was at 37°C and equilibrated with the environment in the tissue culture chamber. A known number (5-10) of 8-cell or blastocyst embryos were transferred directly to the tubes with an elongated, sterile, siliconized pasteur pipet. The tubes were placed upright in the culture chamber with caps

Plate 1: Fluorescing 8-cell BB rat embryo after cryopreservation.

loosened. Temperature, atmosphere and humidity were precisely controlled by the tissue culture chamber and pH was closely monitored throughout the culture period. Eighteen to 24 hours later, the embryos were removed from the culture tubes, and transferred immediately to PBl. They were counted, and their morphological stage recorded.

In Vivo Assay of Viability (Embryo Transfer)

Female Sprague-Dawley recipients were between the ages of 3 months and 1 year. Vaginal smears were preformed on the female and when in proestrus that female was mated with a vasectomized hooded male overnight.

The males were mated three times to ensure they were fertile. Vasectomies were then performed on all hooded (black eyed) males and they were allowed three weeks to recover. They were then used as proven breeders for pseudopregnancy.

Synchronization of the day of pseudopregnancy and the age of the embryo is very important. All recipients were 3 or 4 days pseudopregnant (day 1 being the morning following breeding) for day 4 (8-cell) embryos. For day 5 (blastocysts), the female recipients were 4 or 5 days pseudopregnant. For transplantation, the female recipients were anesthetized with pentobarbital as before. A small flank incision was made just distal to the last rib. A suture was placed through the muscle wall at one end. The ovary and uterus were located by picking up the fatty pad associated with the ovary and gently brought through the incision. Care was taken only to handle the fatty pad. A blunt trocar was carefully placed through the mesentary between the distal tip of the uterus and the fatty tissue. This was used to manipulate the uterus

throughout the transfer. Care was taken not to disturb the ovary, oviducts or uterus. A small number 7 sewing needle was used to make a puncture hole at the distal tip of the uterus, through the wall into the lumen. At the same time a second person collected a known number (3-10) of embryos into an elongated, siliconized pasteur pipet. The embryos were in a minimal amount of media and trapped between 2 air bubbles for identification. A silk suture (# 4.0) was placed approximately 0.7 cm. from the tip of the pipet. The pipet with embryos was carefully placed through the hole into the lumen of the uterus, as far as the suture. The embryos were then gently blown into the uterus. As soon as the second air bubble was at the tip of the pipet, an air lock was placed on the pipet to prevent medium from going into the uterus, and to prevent possible back suction of the embryos. Photographic plate 2 shows the transfer procedure used in this study.

The pipet was held in place for 5-10 seconds and then very carefully removed. After a few minutes, the trocar was removed and the ovary and uterus replaced by pulling up on the suture previously placed in the muscle wall. The incision was closed using a continuous stitch for the muscle layer, and staples were used to close the skin. The rat was kept warm and observed until it regained consciousness. The transplants were checked either at day 14 post-transfer, or allowed to go to term. After 25 days had passed, all transplanted recipients without litters were checked.

Several changes to the transplantation technique were made in an attempt to improve the method. Originally, a 23 gauge needle was used to make the initial puncture through the uterine wall and the embryos

Plate 2: Embryo transfer technique.

were transferred in a sterile elongated pasteur pipet with only one air bubble placed above the donor embryos. At first, the pipet was removed immediately; time was not allowed for the transferred embryos to settle into the uterus.

Statistical Analysis

Yates Corrected Chi Square was used to distinguish significant differences between control and experimental groups for all viability assays (103,104).

IV

RESULTS

Hormonal Induction of Ovulation

Table 1 shows the effect of varying doses of PMSG and HCG on ovulation in the immature BB rat. Twenty IU of PMSG and 15 IU of HCG given 50 to 54 hours apart gave the best results with 76.2% of the embryos ovulated being morphologically normal. With this combination only 9.1 embryos per rat were obtained on isolation. Results were not consistent and hormonal induction of ovulation did not yield more normal embryos than natural mating. Many females were not fertilized or had large numbers of embryos which were not at the correct stage of development. Large numbers of degenerating embryos were also isolated on days 4 and 5 with only a few normal 8-cell or blastocyst embryos. The effectiveness of different lot numbers of both hormones appeared to vary. HCG was also very unstable once dissolved in solution even when stored at -80°C, as a greater percentage of rats ovulated when given freshly prepared HCG than did rats given HCG that had been stored at -80°C for 2 to 3 weeks. For these reasons, embryos were obtained through natural mating for this study.

Natural Mating

Table 2 gives the percentage of morphologically normal BB rat embryos isolated on day 4 and day 5 post mating. Day 4 isolations provided an average of 10.4 8-cell embryos per rat. Forty-four per cent of these embryos were morphologically normal on microscopic examination.

TABLE 1
EFFECT OF VARYING DOSES OF PMSG AND HCG ON OVULATION IN THE IMMATURE BB RAT

PMSG ✚ [IU]	HCG ✦ [IU]	TIME BETWEEN [HOURS]	NUMBER OF RATS	PERCENT NORMAL	AVERAGE # PER RAT
15	10	48 - 50	41	64.65	11.70
15	10	51 - 54	30	58.16	8.36
15	15	51 - 54	10	46.50	7.10
15	20	54 - 59	6	50.00	6.00
20	10	48 - 53	5	29.30	15.00
20	15	50 - 54	18	76.21	9.10
25	10	48 - 59	7	68.00	13.00
25	15	48 - 54	9	64.70	11.30
25	20	50 - 54	5	50.00	9.60

✚ Pregnant Mare Serum Gonadotrophin

✦ Human Chorionic Gonadotrophin

TABLE 2
PERCENTAGE OF MORPHOLOGICALLY NORMAL EMBRYOS OBTAINED THROUGH NATURAL MATING

DEVELOPMENTAL STAGE	NUMBER OF EMBRYOS	NORMAL NUMBER	NORMAL PERCENTAGE	AVERAGE PER RAT
8 - Cell	770	339	44.02**	10.7
Blastocyst	552	475	86.05**	8.35

** Significant at the 0.01 level using Yates Corrected Chi Square.

Day 5 isolations provided an average of 8.35 blastocysts per rat. Eighty-six per cent of these were morphologically normal on microscopic examination. The number of morphologically normal embryos at the time of isolation was significantly more on day 5 than on day 4 at the $p<0.01$ level using the Yates Corrected Chi Square Test for Independence.

The presence of a sperm plug on the morning following mating was not consistent with the presence of fertilized embryos. The majority of female BB rats did not have a plug and yet had morphologically normal embryos in the correct stage of development, where many with a sperm plug had unfertilized oocytes or no embryos at all on day 4 or day 5 isolation. Therefore, the presence of a sperm plug was not used as an indication of fertilization. All female BB rats mated in proestrus were assumed fertilized. Photographic plate 3 shows the stages of the estrus cycle in the female BB rat, as indicated by vaginal lavage. The pictures show the sequential changes in occurrence and appearance of epithelial cells and leukocytes.

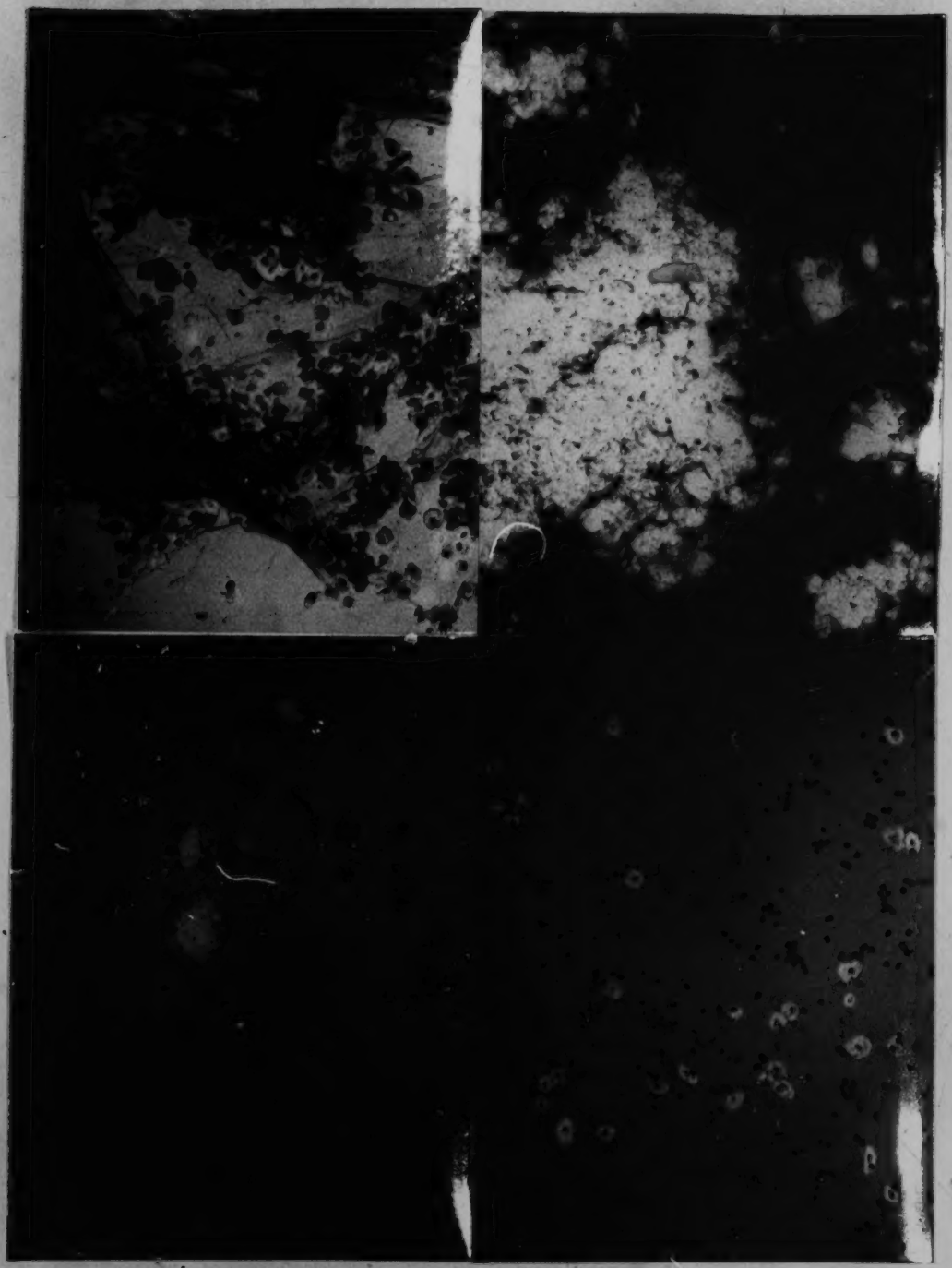

Plate 3: Vaginal milieu picture of the estrus cycle in the female BB rat: a) Proestrus, b) Estrus, c) Metestrus, d) Diestrus.

In Vitro Assays of Viability

A. Fluorescein Diacetate Assay

Table 3 shows both control and experimental groups for 8-cell and blastocyst embryos. Table 4 gives the Yates Corrected Chi Square value for comparisons of the control and experimental groups in Table 3.

Exposure to the cryoprotectant Me_2SO did not affect the survival of 8-cell or blastocyst BB rat embryos as measured by their ability to fluoresce. Survival of 8-cell embryos was significantly reduced when frozen and thawed, irrespective of the freezing protocol followed. There was also a significant decrease in survival when blastocysts were frozen using either protocol #1 or #2. There was no significant difference between the two freezing protocols for 8-cell embryos or blastocysts. Blastocysts frozen slowly to -40°C survived better than 8-cell embryos frozen using the same protocol.

B. Embryo Culture

In all *in vitro* culture assays, development of 8-cell embryos to blastocysts and of blastocysts to expanded blastocysts was the measure of viability.

Table 5 shows the two different culture methods used and their significant differences. Development of BB rat embryos, 8-cell and blastocysts, cultured using the batch method was significantly better than embryos cultured in microdrops under equilibrated paraffin oil. Seventy-two percent of the 8-cell developed to blastocysts and 74% of

TABLE 3
FLUORESCEIN DIACETATE ASSAY
ABILITY OF BB RAT EMBRYOS TO FLUORESCE
AFTER FREEZING AND THAWING

PROTOCOL	DEVELOPMENTAL STAGE	NUMBER OF EMBRYOS ASSAYED	EMBRYOS FLUORESCING NUMBER	EMBRYOS FLUORESCING PERCENT
M. Control ✧	8 - Cell	32	31	96.9
M. Control	Blastocyst	36	36	100.0
Protocol #1 ✚	8 - Cell	117	75	64.1
Protocol #1	Blastocyst	115	93	80.9
Protocol #2 ✣	8 - Cell	76	50	65.8
Protocol #2	Blastocyst	32	24	75.0

✧ Manipulated Control (M. Control): Embryos exposed to Me_2SO but not frozen.

✚ Protocol #1: Slowly cooled (0.25°C/Min) to -40°C; transferred to -196°C; rapidly warmed (200°C/Min).

✣ Protocol #2: Slowly cooled (0.25°C/Min) to -75°C; transferred to -196°C; slowly warmed (18°C/Min).

TABLE 4
CHI SQUARE VALUES FOR MANIPULATED CONTROL, FREEZE PROTOCOL #1 AND FREEZE PROTOCOL #2 ASSESSED USING FLUORESCEIN DIACETATE DYE

COMPARISONS			YATES CORRECTED X^2
M. Control ✧ 8 - Cell	vs.	M. Control Blastocyst	0.004
M. Control 8 - Cell	vs.	Protocol #1 ✚ 8 - Cell	11.597**
M. Control 8 - Cell	vs.	Protocol #2 ✣ 8 - Cell	10.007**
M. Control Blastocyst	vs.	Protocol #1 Blastocyst	6.598*
M. Control Blastocyst	vs.	Protocol #2 Blastocyst	7.934*
Protocol #1 8 - Cell	vs.	Protocol #2 8 - Cell	0.007
Protocol #1 Blastocyst	vs.	Protocol #2 Blastocyst	0.231
Protocol #1 8 - Cell	vs.	Protocol #1 Blastocyst	7.344**
Protocol #2 8 - Cell	vs.	Protocol #2 Blastocyst	0.510

✧ Manipulated Control (M. Control): Embryos exposed to Me_2SO but not frozen.

✚ Protocol #1: Slowly cooled (0.25°C/Min) to -40°C; transferred to -196°C; rapidly warmed (200°C/Min).

✣ Protocol #2: Slowly cooled (0.25°C/Min) to -75°C; transferred to -196°C; slowly warmed (18°C/Min).

* Significant at 0.05 level

** Significant at 0.01 level

TABLE 5
COMPARISON OF CULTURE TECHNIQUES USING FRESH EMBRYOS

TECHNIQUE	DEVELOP. STAGE	NUMBER ASSAYED	PROGRESSED IN CULTURE NUMBER	PERCENT	YATES X^2
Microdrops Under Oil ✲	8 - Cell	95	21	22.00	39.17 **
Batch Culture ✡	8 - Cell	69	50	72.46	
Microdrops Under Oil	Blastocyst	60	5	8.33	36.14 **
Batch Culture	Blastocyst	27	20	74.00	

✲ Method developed by Brinster (10).

✡ Embryos cultured in serological tubes.

** Significant at 0.01 level

the blastocysts developed to expanded blastocysts using the batch culture method. Because of these findings, the batch culture technique was used in all experiments.

Table 6 shows the results for control and experimental groups using batch culture as the in vitro assay. Table 7 shows the Yates Corrected Chi Square value for comparisons of control and experimental groups. Fresh 8-cell embryos developed significantly better in culture than did fresh 8-cell embryos exposed to Me_2SO, as well as better than either freezing and thawing protocol.

There was no significant difference in survival between fresh blastocysts and fresh blastocysts exposed to Me_2SO in the batch culture test of viability. Fresh manipulated blastocysts developed better in culture than blastocysts frozen slowly to -75°C. Blastocysts frozen slowly to -40°C survived as well as either control group.

Blastocysts survived cryopreservation better than did 8-cell embryos. More blastocysts developed in culture than did the 8-cell embryos irrespective of the freeze-thaw protocol used.

Table 8 shows the effect of pH on the development of fresh 8-cell embryos and blastocysts in culture. In initial experiments, the concentration of CO_2 was regulated only by the culture chamber. It was noted that eight-cell embryos developed best with a pH ranging from 7.27 to 7.32. All blastocysts developed to expanded blastocysts at a pH of 7.29 to 7.36. Eight-cell embryos did not develop as well at a pH above 7.34. To better regulate the pH in further experiments, a fyrite gas analyser was used to check the CO_2 concentration. The pH of the medium was checked regularly throughout the in vitro culture period.

TABLE 6
SURVIVAL OF FROZEN - THAWED BB RAT EMBRYOS IN VITRO USING BATCH CULTURE

PROTOCOL	DEVELOPMENTAL STAGE	NUMBER OF EMBRYOS ASSAYED	PROGRESSED IN CULTURE NUMBER	PROGRESSED IN CULTURE PERCENT
Control ✚	8 - Cell	69	50	72.46
Control	Blastocyst	27	20	74.00
M. Control ✧	8 - Cell	29	9	31.03
M. Control	Blastocyst	28	26	92.85
Protocol #1 ✚	8 - Cell	35	11	31.42
Protocol #1	Blastocyst	66	49	74.24
Protocol #2 ✧	8 - Cell	36	8	22.20
Protocol #2	Blastocyst	34	20	58.82

✚ Control: Fresh culture.

✧ Manipulated Control (M. Control): Embryos exposed to Me_2SO but not frozen.

✚ Protocol #1: Slowly cooled (0.25°C/Min) to -40°C; transferred to -196°C; rapidly warmed (200°C/Min).

✧ Protocol #2: Slowly cooled (0.25°C/Min) to -75°C; transferred to -196°C; slowly warmed (18°C/Min).

TABLE 7
CHI SQUARE VALUES FOR CONTROL, MANIPULATED CONTROL, FREEZE PROTOCOL #1 AND FREEZE PROTOCOL #2 ASSESSED USING IN VITRO BATCH CULTURE

COMPARISONS			YATES CORRECTED X^2
Control 8 - Cell	vs.	Control ✚ Blastocyst	0.000
Control 8 - Cell	vs.	M. Control ✧ 8 - Cell	12.949**
Control 8 - Cell	vs.	Protocol #1 ✚ 8 - Cell	14.476**
Control 8 - Cell	vs.	Protocol #2 ✧ 8 - Cell	22.162**
Control Blastocyst	vs.	M. Control Blastocyst	2.304
Control Blastocyst	vs.	Protocol #1 Blastocyst	0.000
Control Blastocyst	vs.	Protocol #2 Blastocyst	0.948
M. Control 8 - Cell	vs.	Protocol #1 8 - Cell	0.055
M.Control 8 - Cell	vs.	Protocol #2 8 - Cell	0.268
M. Control Blastocyst	vs.	Protocol #1 Blastocyst	3.149
M. Control Blastocyst	vs.	Protocol #2 Blastocyst	7.597**
Protocol #1 8 - Cell	vs.	Protocol #2 8 - Cell	0.370
Protocol #1 8 - Cell	vs.	Protocol #1 Blastocyst	15.655**
Protocol #1 Blastocyst	vs.	Protocol #2 Blastocyst	1.825
Protocol #2 8 - Cell	vs.	Protocol #2 Blastocyst	8.295**

✚ Control: Fresh Culture.

✧ Manipulated Control (M. Control): Embryos exposed to Me_2SO but not frozen.

✚ Protocol #1: Slowly cooled (0.25°C/Min) to -40°C; transferred to -196°C; rapidly warmed (200°C/Min).

✧ Protocol #2: Slowly cooled (0.25°C/Min) to -75°C; transferred to -196°C; slowly warmed (18°C/Min).

** Significant at 0.01 level

TABLE 8
EFFECT OF pH ON FRESH BB RAT 8-CELL AND BLASTOCYST EMBRYOS AFTER BATCH CULTURE * FOR 18-24 HOURS

DEVELOPMENTAL STAGE	NUMBER ASSAYED	DEVELOPMENT NUMBER	DEVELOPMENT PERCENT	pH
8-Cell	37	17	45.9	7.10 - 7.13
8-Cell	43	32	74.4	7.27 - 7.32
8-Cell	36	14	38.8	7.34 - 7.44
Blastocyst	8	8	100	7.29
Blastocyst	14	14	100	7.36

* Cultured in BMOC-3 (10) in 5% CO_2 in air at 37°C and 95% humidity.

In Vivo Assay of Viability (Embryo Transfer)

Table 9 shows the results for control and experimental groups for both 8-cell embryos and blastocysts using the in vivo assay of viability, embryo transfer. In all groups control and experimental, the survival rates for transplantation of BB rat embryos into pseudopregnant female recipients were extremely low. Thirteen and a half per cent of blastocysts frozen slowly to -40°C before rapid cooling to -196°C and subsequent thawing, developed into live fetuses after transfer to pseudopregnant females. This freeze-thaw protocol appeared to give the best survival rate as measured by in vivo transfer, however, this may have been due to the large number of embryos included in this group.

Blastocysts frozen slowly to -40°C survived better after in vivo transfer than did 8-cell embryos frozen using the same protocol.

TABLE 9
SURVIVAL OF FROZEN - THAWED BB RAT EMBRYOS IN VIVO

PROTOCOL	EMBRYOS TRANSFERRED NUMBER	STAGE	DEVELOPMENT ★ NUMBER	PERCENT
Control ✚	49	8 - Cell	2	4.08
Control	114	Blastocyst	8	7.00
Protocol #1 ✚	80	8 - Cell	2	2.50
Protocol #1	193	Blastocyst	26 ☥	13.47
Protocol #2 ✣	137	8 - Cell	1	0.73
Protocol #2	8	Blastocyst	0	0.00

✚ Control: Fresh culture.

✚ Protocol #1: Slowly cooled (0.25°C/Min) to -40°C; transferred to -196°C; rapidly warmed (200°C/Min).

✣ Protocol #2: Slowly cooled (0.25°C/Min) to -75°C; transferred to -196°C; slowly warmed (18°C/Min).

★ Day 14 post transfer.

☥ 12 Born live.

V

DISCUSSION

The BB rat provides an excellent animal model for the study of the etiology, pathophysiology and complications of human insulin dependent diabetes mellitus (1-4). Diabetic rats of breeding age must be given daily insulin therapy. The diabetic syndrome also causes reduced fertility and litter size, as well as, insufficient lactation in females (10). With optimal techniques for cryopreservation, *in vitro* manipulation and culture, and *in vivo* embryo transfer, the BB rat strain could be maintained in a low temperature embryo bank at a very low cost. Low temperature storage of this rat embryo would also ensure the strain would not be lost to disease, natural hazards or genetic drift.

The development of *in vitro* and *in vivo* techniques for mammalian embryos has been done largely using the mouse or rabbit. There is very little known about the specific nutritional requirements of the preimplantation rat embryo *in vitro* or of the optimal freezing and thawing techniques for this embryo. *In vivo* embryo transfer in the rat has also been less successful than in the mouse.

Some of the problems presented by the rat embryo will be considered in this study in an attempt to better understand the effects of cryopreservation, *in vitro* manipulation and culture and *in vivo* embryo transfer on the BB rat embryo.

Obtaining Sufficient Numbers of Embryos

Hormonal induction of ovulation is widely used for obtaining preimplantation stages of mouse embryos (12,19,53). This method has not been as successful in the immature rat (13,14,55). In this study, the best results were obtained when 20 IU of Pregnant Mare Serum Gonadotrophin (PMSG) and 15 IU of Human Chorionic Gonadotrophin (HCG) were given 50 to 54 hours apart to 35-45 day old BB rats. Only 76% of these embryos were normal when isolated 4 or 5 days after mating. The average number recovered was 9.1 per rat. Optimal doses for the immature rat have been reported by Rowlands as 30 IU of PMSG and 20 IU of HCG given 48 to 59 hours apart (44). With this regime he obtained 26 embryos per rat. Ovulation occurs in 100% of immature female rats given this hormone regime if under 40 days of age (55). After this age, higher doses of PMSG are necessary to give 90 to 100% ovulation.

Both the amount of hormone and the time between administration of the follicle stimulating hormone and the luteinizing hormone determine the success of superovulation (44,55,56). All three factors were varied in this study and regardless of the regime followed, large numbers of abnormal embryos were observed on both the fourth and fifth day after mating. Even when the doses of hormone and time between were not altered, results were inconsistent, giving large numbers of embryos in a delayed stage of development as well as abnormal cleavage stages. The number of normal embryos isolated varied greatly despite attempts to obtain an optimal regime. Similar observations have been made by a number of investigators. Austin reported an increase in numbers of abnormal embryos as well as unfertilized ova on the second day after

mating in superovulated immature rats (57). More recent studies reveal similar results (105).

In the last few years many studies have considered the problem of increasing numbers of abnormal and unfertilized embryos obtained from superovulated rats. These studies conclude that the failure of preimplantation embryos to develop normally after ovulation is not due to defects in the oocytes, maturation, process of ovulation or fertilization (20,105). Oocytes obtained from superovulated rats become fertilized and develop normally when transferred to fertilized recipients (105). The ovulatory process is normal as large numbers of ova can be recovered the day after fertilization from immature rats after superovulation (22,56). The rate of fertilization is only slightly decreased when eggs are obtained from superovulated females (105). Embryonic death occurs sometime between the second day after fertilization and implantation (20,22,56,57). By the fifth day post fertilization, most investigators report the same number of normal embryos on isolation as would be found in a rat mated naturally (21,106).

The reasons for these large numbers of abnormal embryos and few normal ones in superovulated immature rats has been attributed mainly to an unsuitable maternal endocrine environment (21,106,107). Large nonluteinized follicles have been observed in these immature rats after administration of 40 IU of PMSG (56,107). These persist for several days and are thought to be the source of elevated levels of serum estradiol (107). It has been shown that the levels of estradiol observed in these rats is detrimental to embryo survival (106). Another contributing factor is the fact that ovulation occurs over a prolonged

period of time in superovulated rats and mice (56,107,108). Many oocytes are ovulated prematurely, and enter the uterine environment before it is receptive to embryos. As well, the incidence of polyploidy and other chromosomal anomalies in fertilized ova increase following superovulation (109).

Superovulation can be induced in immature rats, however, it may be best to use natural mating when embryos beyond the 2-cell stage are required. A very recent report shows a significant decrease in viability of frozen thawed mouse embryos obtained from superovulated females compared to embryos obtained through natural mating (11).

In this study, natural mating provided a high percentage of normal blastocysts on day 5 post fertilization with an average of 8.35 embryos recovered per rat. On the fourth day following fertilization there was a larger percentage of abnormal embryos isolated, but an average of 10.7 were recovered from each female. The reason for this larger number of abnormal embryos found on day 4 is not fully understood. One explanation may be that the 8-cell embryos may have been in a particular stage of mitosis when isolated and this stage was less stable in vitro. Several 8-cell embryos appeared to contain a condensed cytoplasm, although their blastomeres were all uniform in size. None of these developed in fresh culture, and were therefore assumed abnormal at the time of isolation. Very few abnormal blastocysts were isolated on day 5, therefore, it may be that some of the abnormal 8-cell embryos would develop into normal blastocysts if left in the maternal environment. Natural mating provided a reliable and simple means for obtaining sufficient numbers of embryos in the BB rat. Females were mated prior

to the onset of diabetes in most cases (90 to 120 days of age), therefore, this did not pose a problem in this study.

Embryo Isolation and In Vitro Manipulation

The present study focussed on the effects of various in vitro and in vivo manipulation procedures on 8-cell or blastocyst BB rat embryos. In the initial experiments all preimplantation stages of these embryos were collected to familiarize the investigator with handling techniques and morphological appearances of the BB rat embryo. One-cell fertilized ova were obtained on the day following fertilization designated as day 1. Two-cell embryos were recovered on day 2, and 4-cell and some 2-cell were recovered on day 3. Eight and sixteen cell embryos were recovered on day 4 and morula and blastocysts on the morning of day 5. Fertilized ova through to 8-cell were recovered from the oviducts and morula and blastocysts from the uterus. Occasionally, a few 8-cell embryos were recovered from the uterus and morula from the oviducts late on day 4.

Brinster reported on the time-line for development of the preimplantation mouse and rat embryo (25). He reported blastocysts entering the uterus between day 3 and 4 in the rat which was not observed in this study.

The time of day appeared to determine the success of recovery of 8-cell and blastocyst embryos in the BB rat (data not shown). The most appropriate time of day for collection of 8-cell embryos was between 2 and 4 pm on day 4. Isolations in the morning of day 4 provided embryos in the 4-cell stage as well as the 8-cell stage. Isolations later than

4 pm resulted in collection of 8-, 16- and 32-cell embryos. This varied from animal to animal but isolations in the afternoon of day 4 provided consistent recovery of 8-cell embryos. When blastocysts were required, isolations between 8 and 10 am on day 5 gave the best results. Depending on the animal, isolations after 2 pm on day 5 were often nonproductive, although implantation is not thought to occur until later in the afternoon (25).

To ensure maximum recovery of embryos both the oviduct and 1 cm of the uterus were flushed when 8-cell embryos were collected and the entire oviduct and uterus when blastocysts were required. Repeated flushing of the remaining portion of the uterus on day 4 yielded no embryos.

The most appropriate preimplantation stage of development of the BB rat embryo for in vitro manipulation was the blastocyst. A higher percentage of normal blastocysts were recovered on isolation. Fresh blastocysts also developed better in culture than did fresh 8-cell embryos. Wood and Whittingham reported better results when rat blastocysts were cultured, cryopreserved or transferred to pseudopregnant females (14).

Degenerative embryos with condensed cytoplasm and cloudy contents were often observed on day 4 along with normal 8-cell embryos. Degeneration of rat embryos has been described in detail by Defrise (40). Darkening and clouding of the cytoplasm is characteristic of degenerating embryos. Although none of these embryos were considered in any of the data collected, they did not fluoresce when tested with the FDA assay, nor did they develop in culture. Other abnormalities observed in day 4 embryos included embryos with blastomeres of varying

sizes; known as fragmentation (58), and embryos in a retarded developmental stage, 4- or 6-cell. The normality of 16-cell embryos was difficult to determine on microscopic examination, and these were also recovered along with 8-cell embryos. None of these abnormalities were seen on day 5 in morula or blastocysts, although occasionally 16-cell embryos were recovered along with morula or blastocysts.

The handling medium used was developed by Dulbecco and Vogt (64) and modified by Whittingham and Whitten (65). The pH was stable in air at 7.2 to 7.32 and embryos did not show any change in morphological appearance when held for up to 2 hours at room temperature in this medium. Whittingham obtained better survival when mouse and rat embryos were held at a constant room temperature prior to transfer into recipients than when the embryos were moved in and out of a 37°C culture chamber (31). This only applied if holding times were less than 3 hours.

Cryopreservation

Mouse embryo cryobanking is a fairly common practice (11,12). Some 500 strains are preserved in one lab alone (11). Rat embryos have been successfully cryopreserved, but survival rates are low (13,14). Two freeze-thaw protocols were investigated in this study. Dimethyl sulfoxide at a concentration of 2 M was used as the cryoprotective agent. Embryos exposed to Me_2SO but not frozen were not affected when tested using the FDA assay. Eight-cell embryos showed a significant decrease in their ability to develop in culture when exposed to the cryoprotectant but not frozen. Dimethyl sulfoxide has been the solute

of choice for protecting against slow freezing injury in the rat embryo (13,14). Neither of these investigators reported any detrimental effect of Me_2SO on viability post-thaw.

Both addition and dilution of Me_2SO was carried out at room temperature. Many investigators add the cryoprotectant at 0°C (13,29,70) and few at room temperature (14). Schnieder suggests that exposure to the cryoprotectant is more efficient at room temperature since the permeation of a solute into the blastomeres is dependent not only on the permeability coefficient of the cell membrane but also on the temperature coefficient of that permeability (78). Addition at 0°C may not allow sufficient permeation to afford protection during slow cooling.

Both in vitro and in vivo tests of viability were used to assess the effects of freezing and thawing on the BB rat embryo. The Blastocyst stage survived best whether embryos were frozen slowly to -40°C or -75°C before rapid cooling to -196°C in all assays of viability.

Fluorescein diacetate provides a rapid initial method of assessing viability. Using this assay the viability of both 8-cell and blastocyst embryos was significantly reduced after cryopreservation. There was however, no difference between the freezing protocols as measured by this assay. Blastocysts survived slow cooling to -40°C significantly better than did 8-cell embryos.

In vitro culture is a more stringent test of viability. Development in culture was only considered positive if 8-cell embryos developed into morula or blastocysts, and blastocysts or morula expanded within 24 hours. In some experiments blastocysts which had developed in

vitro from 8-cell were held in culture for an additional 18 to 24 hours to observe expansion and occasionally, loss of the zona pellucida.

Fresh control embryos developed significantly better than did any of the other experimental groups. The addition and dilution of Me_2SO had a marked effect on survival of BB rat 8-cell embryos. There was no difference between the two freezing protocols for the 8-cell embryos as measured by in vitro embryo culture.

Survival of 8-cell BB rat embryos was significantly reduced (31%), regardless of the freeze thaw protocol. This may be attributed to the apparent adverse effects of 2 Me_2SO, as fresh 8-cell embryos did not develop well in culture after exposure to this concentration of Me_2SO. Whittingham reported 61% of 8-cell rat embryos developed into blastocysts after slow cooling to -80°C and slow thawing (13).

Blastocysts survived both freeze-thaw protocols significantly better than 8-cell embryos. When frozen slowly to -40°C prior to transfer to -196°C, and subsequent rapid thawing, blastocysts developed as well as (74%) either control groups in culture. Survival of blastocysts frozen slowly to -75°C was lower than those exposed to Me_2SO alone and then assessed in culture.

In vivo transfer of the BB rat embryo after freezing and thawing gave very low survival rates regardless of the protocol followed. Again, the best results (13.5%) were obtained when blastocysts were cooled slowly to -40°C prior to transfer to -196°C and then transferred to pseudopregnant recipients after rapid warming. The large number of embryos included in this group may have biased these results. No blastocysts frozen slowly to -75°C prior to rapid freezing to -196°C and

subsequent slow thawing survived after embryo transfer, however, only a few embryos were included in this group.

The low success of embryo transfer after cryopreservation was attributed to the lack of success of the transfer technique and not the freeze-thaw protocols as very poor results were obtained in control transfers as well.

One other study considered the same two freeze-thaw protocols in 1980 (14). These investigators used 1.5 M Me_2SO as the cryoprotective additive and a faster warming rate for embryos cooled slowly to -40°C. Wood and Whittingham only looked at *in vivo* transfer as a measure of viability. Their results were slightly better when slow cooling was stopped at -80°C (21%). They also considered different slow cooling rates but made no conclusive remarks. These investigators only considered the rat blastocyst stage for cryopreservation.

Viability as measured by *in vitro* assays showed no difference between the two freezing protocols employed in this study. Since slow cooling to -40°C prior to transfer to liquid nitrogen for storage and subsequent rapid thawing is less time consuming, this protocol may be the better of the two for the BB rat embryo. The blastocyst is the more stable stage of development for cryopreservation in the BB rat as measured by these *in vitro* assays.

BB rat embryos were stored in liquid nitrogen for up to 4 years. Blastocysts stored at -196°C for 4 years survived well on thawing (71.43%) as measured by in vitro culture. Mouse embryos have been stored for up to 11 years without detrimental effects (12). The only biological reaction which takes place at -196°C, background radiation, is not a significant problem (85). There are however, a few practical

problems which could affect the viability of the stored embryos. Low levels of liquid nitrogen or total failure of a storage container are realistic possibilities (12), and there is the remote possibility of cross contamination between materials in the liquid nitrogen tank especially if vials are not sealed properly (11). In mouse embryo cryobanking, mutant strains tend to survive less well than do hybrid strains (12). The health status of the thawed embryos will depend on that of the foster mother and the colony where they will be introduced (11).

A very recent technique, vitrification, has been developed as a new method of cryopreserving mouse embryos (110). Vitrification involves exposure of the embryos to very high concentrations of a mixture of cryoprotectants. The vitrifying solution (VSI), contains Me_2SO, acetamide and propylene glycol. Toxicity of these cryoprotectants is reduced by decreasing the time and temperature of exposure to the embryos. Mouse embryos exposed to 87.5% VSI survive very well (94.7%) when they are held for 20 min at 4°C in this solution. The embryos dehydrate to the degree that no ice forms when they are subsequently cooled at very rapid rates (200°C/min) to -196°C. They also survive rapid dilution of the cryoprotectants after rapid thawing (300°C/min). When slow cooling of mouse embryos are observed under the cryomicroscope no intracellular freezing occurs if slow cooling is stopped at -40°C and the embryos are cooled rapidly to -196°C (18). The cytoplasm is thought to form a glassy state or vitrify in this procedure as well.

Viability Assays

A. Fluorescein Diacetate Assay

The use of fluorescein diacetate as an initial test of viability of frozen thawed embryos has not been widely used. In this study, embryos appearing morphologically normal on thawing always fluoresced. This test did not reveal the loss of viability of 8-cell embryos exposed to the cryoprotectant Me_2SO. Although a high percentage of 8-cell BB rat embryos fluoresced (65%), the ability of 8-cell BB rat embryos to develop in culture after exposure to Me_2SO and freezing was significantly reduced (31%). The ability of blastocysts to fluoresce and develop in culture was similar in all protocols considered.

Since this test is only a measure of the integrity of the plasma membrane and eterase enzyme activity (92), it is possible that 8-cell embryos lose their viability after exposure to Me_2SO or post-thaw as time progresses or that other cell mechanisms are damaged beyond repair during the freezing or thawing process. Incubation of the embryos with FDA after *in vitro* manipulation or cryopreservation may give a better indication of viability.

Mohr and Trounsen (92) studied the effect of FDA and UV light on 2-cell mouse embryos. Neither of these factors affected the ability of the mouse embryo to develop in culture or to develop *in vivo* after transfer to a foster mother. This technique could be used as an initial nonintrusive measure of viability after freezing and thawing before subsequent transfer to a pseudopregnant recipient.

B. Embryo Culture

In vitro culture of mammalian embryos is an established test of viability. Most studies employ Brinsters technique (15) of culturing embryos in microdroplets of medium under paraffin oil (13,14,29). A few studies in the 1950's cultured mouse embryos in serological tubes in a small amount of medium (26,41,42). Both techniques were attempted in this study as initial results with Brinsters method gave very low survival rates for control groups. The test tube or batch method was very successful and technically much easier. Fresh 8-cell and blastocyst embryos developed significantly better with the batch method of culture. Brinster suggests that the main disadvantage of the batch method is, loss of embryos on recovery from the test tubes (111). This was never a problem in this study. The culture chamber atmosphere was saturated with water vapor at 95% humidity and was precisely controlled. There was no problem with evaporation and subsequent increased osmotic pressure of the medium which would be fatal to the embryos (24).

The main advantages of the microdrop technique are that small amounts of medium are used and embryos can be quickly identified in the same culture dish. The oil also prevents evaporation (15). There are a few reasons why the microdrop culture method may have failed. The quality of paraffin oil is important. Some paraffin oils contain an impurity which results in the formation of a thin, viscous film between the culture medium and the mineral oil and development of embryos is retarded when this film is present (37). Some components of the culture medium, such as pyruvate, are soluble in mineral oil. These components

will equilibrate with the oil and this may interfere with development of the embryos (37).

The survival of fresh 8-cell embryos using the batch method of culture was similar (72.5%) to those reported for 8-cell rat embryos cultured under oil (79.0%) by Mayer and Fritz (27). These investigators used Biggers, Whitten and Whittingham medium substituting BSA with FCS. Folstad, Bennet and Dorfman reported a higher percentage (90%) of 8-cell rat embryos developing to morula or blastocysts after 24 hours in culture using Brinsters medium (BMOC-3) (28). Wood and Whittingham tested various culture media using 8-cell rat embryos and obtained best results (44%) with medium based on Tyrodes solution (14). Better results were achieved using Brinsters medium (BMOC-3) in this study than in Wood and Whittingham's study.

In vitro culture of the BB rat blastocyst was a good measure of viability. Seventy-four per cent of blastocysts cooled slowly to -40°C and 59% cooled slowly to -75°C before transfer to -196°C expanded in culture in 24 hours on thawing. Hatching from the zona pellucida was observed in 33% of rat blastocysts cultured from the morula or blastocyst stage in 20 to 25 hours. Hatching occurred in control and experimental groups. Mayer and Fritz suggest that the substitution of FCS for BSA may initiate hatching (27), however, the medium used in this study only contained BSA. Wood and Whittingham did not observe hatching from the zona in their study (14).

The effect of hydrogen ion concentration on embryo development has not been extensively studied. Brinster points out that when considering the effect of pH, the concentration of energy source and CO_2 content of the medium are also taken into account (24). The availability of energy

sources such as pyruvate and lactate to the developing embryo is related to the pH of the medium. The lower the pH, the lower the concentration of substrate necessary for optimal development. The CO_2 concentration also directly affects the pH of the medium since a bicarbonate buffer is necessary to support mammalian embryo development.

Although an in depth study of the effects of pH on BB rat embryo development was not undertaken in this study, several observations were made. The concentration of CO_2 (5%) was electronically controlled by the culture chamber. Even with this control, a constant monitoring of the pH of the culture medium was necessary. A fyrite gas analyzer was also used as a second control in maintaining an optimum pH range for development of the BB rat embryo in vitro.

The BB rat embryo is very sensitive to changes in pH. Fresh 8-cell embryos developed best at a pH of 7.27 to 7.32. A more basic pH was detrimental to development of the 8-cell embryo. Blastocysts developed well at a pH of 7.36 where 8-cell embryos did not. This factor was the most critical in determining the success of in vitro cultivation of the BB rat embryo. For viability assays, only embryos cultured at a pH ranging from 7.27 to 7.32 were considered in the final data.

In Vivo Embryo Transfer

In vivo transfer of frozen-thawed embryos is the ultimate test of survival. Transfer of rat embryos has not been as successful as for the mouse embryo. Many factors play equally important roles in determining the success of this technique. The induction of pseudopregnancy, normality of donor embryos, synchrony between the developmental stage of

donor embryos and stage of pregnancy of the recipient uterus and the technical aspects of performing a successful transfer must all be perfect for success (32-34).

In this study, survival of the BB rat embryo in vivo was very low. Only 4% of fresh 8-cell and 7% of fresh blastocysts developed into normal fetuses. For these reasons this test for viability on frozen-thawed BB rat embryos did not depict the effects of the different cryopreservation protocols but rather the effects of the embryo transfer technique itself.

The BB rat 8-cell and blastocyst embryo can be successfully cooled slowly to -40°C and then stored at -196°C before rapid warming, as 2 out of 80, 8-cell embryos and 26 of 193 blastocysts did develop into live fetuses. Twelve rats were born live and developed normally after being frozen for 15 days to 4 months. Photographic plate 4 shows a frozen-thawed BB rat blastocyst. Photographic plate 5 shows 21-day old BB rats previously frozen for 2 weeks. Only two papers report the successful transfer of rat embryos after cryopreservation. Whittingham transferred 8-cell rat embryos after slow cooling to -80°C and subsequent storage in liquid nitrogen, and slow warming. Nine per cent of these developed in vivo after slow thawing (13). Wood and Whittingham studied two different freeze-thaw protocols and reported slightly better survival of rat blastocysts transferred to recipients on thawing using the same freeze-thaw protocol as Whittingham (14). Twenty-one per cent of the blastocysts frozen with this protocol developed in vivo where only 14 % of blastocysts cooled slowly to -40°C before storage in liquid nitrogen developed in vivo when thawed rapidly.

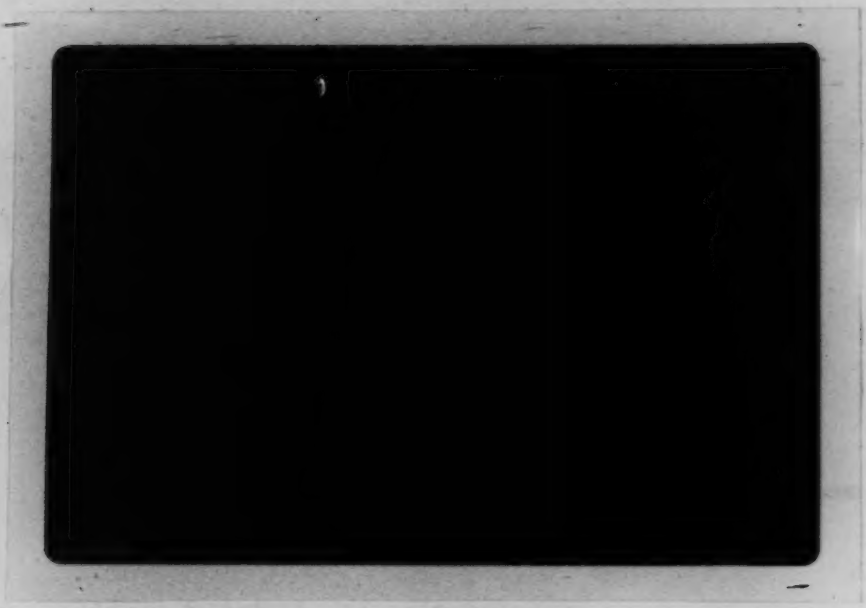

Plate 4: BB rat blastocyst after freezing and thawing.

Plate 5: 21-day old BB rats previously stored in liquid nitrogen at the blastocyst stage for 2 weeks.

An interesting observation in their study was that more embryos warmed rapidly appeared morphologically normal (58%) than embryos warmed slowly (49%) (14). In the present study, a much larger number (75-80%) of embryos appeared normal on thawing.

There are several possible reasons for such low success in this technique. The Sprague-Dawley female recipients may not have been in a state of pseudopregnancy. These females were mated with vasectomized hooded males when vaginal smears showed proestrus. Repeat vaginal smears were done on 2 days following mating to note the presence of a continuous state of diestrus which indicates pregnancy or pseudopregnancy (59). In some cases, smears were checked again on day 4, as cycling females would again be in proestrus. If females showed any other stage of the estrus cycle they were not used as recipients. Many of these rejected females did not begin cycling regularly for up to 3 weeks. Since rodent require physical stimulation to induce pseudopregnancy (31,32), the physical trauma incurred during the embryo transfer may upset this state of pseudopregnancy. One way to determine whether the recipient is still in the state of pseudopregnancy would be to monitor serum progesterone levels before and after the transfer procedure. The cycling of this hormone would not continue if pseudopregnancy persisted.

In all transfer experiments, control or experimental, only morphologically normal BB rat embryos were transferred. On thawing abnormal embryos were included in the data, but not transferred.

Synchrony of the state of pseudopregnancy and the developmental age of the embryo was varied in control and experimental groups. Eight-cell embryos were transferred to day 3 and 4 pseudopregnant females. Dickman

and Noyes suggest transfers on day 3 of pseudopregnancy are never successful (34). In this study, although all survival rates were low, asynchronous transfers appeared to be better for 8-cell embryos (4 live fetuses and 2 implant sites) than synchronous transfers (1 live fetus). Blastocysts were transferred on day 4 or 5 of pseudopregnancy and survived equally as well. Transfers done late on day 5 are usually less productive (34), therefore, day 5 transfers were always performed before 2 pm in this study.

Asynchronous embryo transfers may be advantageous if embryos have suffered developmental delay because of *in vitro* manipulations such as cryopreservation (31). Freezing and thawing may delay resumption of normal metabolic processes. Frozen-thawed rabbit embryos survive better when transfers are asynchronous (112). Some investigators believe that post-thaw culture allows cryopreserved embryos time for recovery prior to transfer (12). This technique was tried in this study with a small group of embryos (data not shown). The effect of culture on the survival of frozen thawed rat embryos and subsequent transfer was also considered in this study. Fresh 8-cell embryos were cultured for 24 hours and the resulting blastocysts frozen slowly to -40°C, stored in liquid nitrogen and rapidly warmed before transfer. Four out of 7 of these embryos developed into live fetuses. Allowing blastocysts to recover from the freeze-thaw procedure by culturing for 18 hours gave poor results. No live fetuses resulted after synchronous transfer, but degenerating implant sites were present on day 14 post-transfer. Resumption of development may begin in culture after thawing and may result in a less desirable candidate for implantation. Late mouse

blastocysts do not survive embryo transfer as well as early blastocysts (34).

A major cause of failure may have been the actual transfer technique. Experience is definitely important and for this reason some of the early attempts at transfer may have failed because of lack of experience. Trauma to the uterus is unacceptable and usually causes loss of the entire transfer (32). Injection of air may also have been a cause of failure (113). In this study, embryos were transferred in a minimal amount of medium trapped in the very tip of a drawn siliconized, sterile pipet between two tiny air bubbles. Without these air bubbles there was less control over the amount of medium entering the uterus. Mouse embryos survive best when minimal amounts of medium are transferred with the embryos (58).

After transfer of the embryos the pipet was held in place for 5 to 10 seconds to allow the embryos to settle. This was done to reduce the possibility of the embryos being drawn back through the puncture hole immediately after removal of the pipet.

Lack of sterility may also have been a problem in this study. Precautions were taken to ensure sterility, such as, sterilization of all media, glassware and surgical instruments used, however, the early luteal phase uterus is particularly susceptible to infection and in the rabbit this is the principle cause of failure (32).

It is obvious that further investigation into the area of <u>in vivo</u> transfer of the rat embryo is necessary. There are very few studies on the effects of <u>in vitro</u> manipulation followed by embryo transfer in the rat. Since the establishment of an embryo cryobank for the BB rat

depends on the success of *in vivo* transfer, further research must be focused on this area.

VI

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

(1) Diabetic BB rat embryos can be successfully frozen and thawed. Normal young develop after transfer of thawed 8-cell embryos and blastocysts into pseudopregnant recipient females.

(2) The ability of the 8-cell BB rat embryo to develop in culture was significantly reduced when exposed to 2 M Me_2SO, the cryoprotective agent used in this study.

(3) There was no significant difference in survival as measured by in vitro assays, of the BB rat 8-cell or blastocyst stage when slow cooling was stopped at either -40°C or -75°C before rapid cooling to -196°C and subsequent rapid or slow warming respectively. Since slow cooling to -40°C and rapid thawing is less time consuming and is equally as effective in cryopreserving BB rat embryos, the author feels this is the better freeze-thaw protocol.

(4) The BB rat blastocyst survived both freezing the thawing protocols significantly better than the 8-cell stage when tested with either in vitro assays or in vivo embryo transfer. The blastocyst appears to be the better preimplantation

developmental stage for *in vitro* manipulation, cryopreservation and embryo transfer in the BB rat.

(5) Superovulation with Pregnant Mare Serum Gonadotrophin followed by Human Chorionic Gonadotrophin is not a reliable method for obtaining large numbers of normal embryos in the BB rat.

(6) The ability of BB rat blastocysts to fluoresce in the presence of the dye, fluorescein diacetate, was a good indication of viability and agreed well with *in vitro* culture results.

(7) The *in vitro* culture technique, microdrops under oil, used by most investigators for mammalian embryo culture gave very poor results in this study. Embryos cultured in serological tubes (batch culture), in a small amount of Brinsters' medium (BMOC-3), survived significantly better than those cultured using the conventional method.

(8) Eight-cell BB rat embryos appeared more sensitive to changes in pH than the blastocyst stage. The optimal range of pH for the successful culture of these stages of the BB rat embryo was 7.27 to 7.32.

(9) *In vivo* transfer of the BB rat embryo gave very low survival rates in all groups, control and experimental, in this study. The blastocysts frozen slowly to -40°C, stored in liquid nitrogen and thawed rapidly survived significantly better

after embryo transfer than any other protocol. Despite several changes to the original method of embryo transfer, survival remained low. It was concluded that the low survival of BB rat embryos transferred to recipient females was not a failure of the freeze-thaw technique but of the method of *in vivo* transfer itself.

Recommendations

(1) Development of BB rat 8-cell embryos in culture is markedly reduced after exposure to 2M Me_2SO. This concentration may be toxic to the 8-cell BB rat embryo or the method of addition and/or dilution used in this study may be detrimental to the viability of this stage of development. Rat embryos survive exposure to 1.5 M Me_2SO (13,14). Further investigation into the effects of varying concentrations of Me_2SO and method and temperature of addition and dilution on the survival of the 8-cell BB rat embryo is necessary to alleviate this problem. Both glycerol and ethylene glycol have been used successfully as the cryoprotective additive for the mouse embryo (74), and ethylene glycol for the rat (116). Perhaps the 8-cell stage will tolerate the presence of a cryoprotective solute such as glycerol or ethylene glycol better than Me_2SO.

(2) The FDA assay provided a rapid non-intrusive means of assessing initial viability of frozen thawed BB rat blastocysts. This assay is less time consuming than the *in vitro* culture test and has no detrimental effect on further development (92). FDA may be a more efficient means of initial *in vitro* assessment prior to the use of further treatments such as *in vivo* embryo transfer.

(3) The method of batch culture for the BB rat embryo gave significantly better results than the conventional method for

cultivating mammalian embryos in microdrops under oil. The author found this technique to be less time consuming and recovery of cultured embryos from the serological tubes was never a problem. This technique may provide more satisfactory results for rat embryo culture.

(4) In vivo embryo transfer of the BB rat embryo gave very low survival rates. The rat embryo does not survive as well as the mouse in embryo transfer experiments. There is a need for further investigation into the method of inducing pseudopregnancy in recipient females. The fertility of the recipient varies from strain to strain and the most suitable strain for a particular experiment needs to be assessed to improve the success of this technique. Uniparous recipients or genetic marker recipients that are already pregnant may provide more suitable recipients for rat embryo transfers. The technique of embryo transfer may be at fault. It is possible that the state of psuedopregnancy is disturbed in some way during the surgical transfer itself. Different transfer techniques may improve results.

(5) Since the embryo transfer technique has been adopted for the rat from methods developed in the mouse model, it may be necessary to attempt this technique using the mouse model before using the rat model. This would ensure that failure of the proceedure is not due to the actual transfer technique

itself. Once established in the mouse model, the method could then be carried over into the rat model.

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